



PHD

**MSX-2, calcitriol and the control of differentiation in adult human bone-derived cells**

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MSX-2, CALCITRIOL AND THE CONTROL OF DIFFERENTIATION IN ADULT  
HUMAN BONE-DERIVED CELLS

submitted by Julie Michelle Brown

for the degree of PhD

of the University of Bath

1998

A handwritten signature in black ink that reads "Julie Brown". The script is cursive and fluid, with the first letter of each name being capitalized and prominent.

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## Abstract

In the developing organism, interactions between bone morphogenetic proteins (BMPs), homeodomain-containing proteins (including MSX) and fibroblastic growth factors stimulate and maintain the outgrowth of the limb. As endochondral and intramembranous ossification progress, members of the transforming growth factor-beta superfamily, including BMPs, induce expression of MSX genes prior to induction of osteogenic markers. The coincident expression of MSX and BMP genes by cells at bone forming sites, for example in craniofacial structures, and at remodelling sites in the adult, make them excellent candidates for study in an attempt to understand the regulation of the differentiation of osteoblastic cells. In this project, in adult human bone-derived cells, consistent regulation of MSX-2 by BMPs was shown.

The pleiotropic steroid hormone metabolite, calcitriol, has a fundamental role in the control of bone remodelling in the adult. The mechanisms whereby it regulates the cascade of gene expression in the differentiation of osteoblasts therefore are of great interest. In the following investigations, stimulation of the expression of osteoblast-related genes was seen to be consistent. Its direct regulation of osteocalcin gene expression was confirmed, but the control by calcitriol of mRNA expression of BMP-2, BMP-4 and MSX-2 was seen to involve indirect mechanisms.

The presence of osteogenic factors in bone, such as transforming growth factor-beta, BMPs and calcitriol, and their association with the mineralised extracellular matrix strongly suggest a role in the expression of osteoblast-related genes, alone or in combination with other factors. Regulation of osteoblast-related gene expression in osteosarcoma-derived cells and in osteoblastic cells by osteogenic agents was seen to be complex and modulated by other factors, including cell density and cell-matrix interaction. Evidence suggests that the effects of these agents are cell maturation-specific and differ depending upon the derivation of the cells.

In summary, the regulation of osteoblast-related gene expression in adult human osteoblastic cells depends on cell density, maturation state and derivation, and upon the dose of agent(s) used. As in morphogenesis and development, in these adult human osteosarcoma- and bone-derived cells, MSX-2 expression was seen to be consistently regulated by BMP-2 and -4, and by calcitriol. Calcitriol and the synthetic glucocorticoid dexamethasone were seen to respectively enhance the maturation and differentiation of osteoblastic cells, and to alter their pattern of gene expression in a cell maturation stage-specific manner.

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## **Chapter 1**

### **INTRODUCTION**

## 1.1 THE ANATOMY OF BONE

The skeleton has three main roles in the organism: it acts as a support and site for muscle attachment, it protects vital organs and bone marrow, and it is a reservoir of mineral ions, particularly calcium and phosphate. It also maintains blood ion concentrations and acts as a reservoir for growth factors and hormones which can be liberated as required by bone resorbing cells (osteoclasts). In the adult, resorption is coupled to bone formation such that bone is in a constant state of flux and is continuously removed by osteoclasts, then replaced by bone-forming cells (osteoblasts) (see figure 1.1, after Baron, 1990).

There are two kinds of bone in the skeleton: flat bones such as the skull bones, scapula and mandible, and long bones, which include the femur and humerus. There are also two types of bone structure: hard cortical bone which is found in the shafts of the long bones surrounding the marrow cavities and spongy, trabecular or cancellous bone, which consists of a delicate interlacing network, the trabeculae, surrounding cavities containing red or fatty marrow. This latter kind of bone is found in the vertebrae, in most of the flat bones and in the ends of the long bones (Vaughan, 1975).

The main constituent of bone is the extracellular matrix which is composed primarily of collagen fibres, crystals of hydroxyapatite and a ground substance made up of proteoglycans and glycoproteins. These latter two compounds are strongly anionic and have a high ion-binding capacity: these properties are thought to be important in the calcification process and in fixing hydroxyapatite crystals to collagen fibres (Baron, 1990).

Bone formation occurs in the organism during embryonic development, growth, remodelling, fracture repair and when induced experimentally. This complex process is dependent upon the proliferation and differentiation of osteoblast precursors and upon the activity of the fully differentiated osteoblasts, which are primarily responsible for the synthesis, organisation and deposition of an extracellular matrix which becomes physiologically mineralised during bone formation, modelling and focal turnover throughout life. Multipotent stem cells present in the bone marrow stroma can also be induced to differentiate into other cell types, including myoblasts and adipocytes (see figure 1.2, after Bruder et al, 1994).

Osteoblasts synthesize a range of factors including collagen, alkaline phosphatase, bone morphogenetic proteins and osteocalcin (see figure 1.3, after Aubin et al, 1993) and are capable of co-ordinating the temporal and spatial relationships between necessary constituents for the calcification of bone. This calcification process is partnered by hydroxyapatite, an efficient adsorber for many different proteins, which sequesters components from the circulatory system and prevents escape of locally synthesized factors.

In vitro systems have been devised to allow the study of osteoblast function at the cellular level. Human osteoblast-like cells can be obtained following outgrowth

from adult trabecular bone fragments (Beresford et al, 1986) and such cells have been found to display characteristics of osteoblasts including high alkaline phosphatase activity, production of type I collagen and response to factors such as  $1\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> (calcitriol) and bone morphogenetic proteins (BMPs).

## 1.2 BONE FORMATION

### Endochondral ossification

Bone grows in diameter by apposition of new bone on existing bone surface and in length by endochondral ossification (see figure 1.4) (Vaughan, 1975). In embryonic and adolescent long bone development, the cartilage template laid down at the sites of prospective bone is gradually replaced by bone: initially, metaphyseal growth plate cartilage becomes hypertrophic, then the matrix is calcified and resorbed. Marrow-derived mesenchymal stem cells migrate to these resorption sites where they differentiate along the osteoblastic pathway and produce bone matrix. New bone spicules are orientated on the surface of the resorbing hypertrophic cartilage according to vasculature. Then, following the interactions of resorption, synthesis and mechanical forces, the spicules are remodelled into mature weight-bearing bone (Bruder et al, 1994).

Hypertrophic chondrocytes can transdifferentiate into osteoblast-like cells (Roach et al, 1995), characterised by the expression and synthesis of alkaline phosphatase, osteonectin, osteopontin, bone sialoprotein and type I collagen (Fedarko et al, 1992) and responsiveness to hormones and growth factors (Pfeilschifter et al, 1993). These and cells derived from the bone marrow deposit type I collagen on the surface of eroded cartilage to begin the process of endochondral ossification (Galotto et al, 1994).

### Bone repair and regeneration

After breaking a long bone, a group of cells which includes mesenchymal stem cells is drawn to the fracture site to form the repair blastema. If the fracture is mechanically stable, vasculature and bone formation proceeds; if the fracture is unstable, cartilage is produced, then periosteal bone formation occurs to provide a rigid framework, and vascularisation takes place. The cartilage hypertrophies and is replaced by marrow, then bone: lack of vascularisation fosters cartilage formation (Bruder et al, 1994).

Bone substitutes consisting of ceramics, hydroxyapatite and titanium have been used to repair bone with varying degrees of success (Holmes et al, 1987; Wolff et al, 1994; Zambonin and Grano, 1995). The characteristics of material implanted which affect the efficiency of repair are composition, topography, roughness and surface energy of the implant (Schwartz and Boyan, 1994), upon which factors the proliferative ability or physiology of interacting cells may depend (Hunter et al, 1995).

### Mechanical loading

In the adult, mechanical forces regulate bone formation: periosteal bone formation is increased following loading of the bone with an external force. In rat tibia, this was achieved by an initial increase in cell proliferation as shown by increased transcription of transforming growth factor-beta (TGF-beta), c-fos and insulin-like growth factor (IGF-II) with a synchronous decrease in differentiation markers such as alkaline phosphatase, osteopontin and osteocalcin (Raab-cullen et al, 1994). Similarly, mouse calvarial cells mechanically loaded by intermittent hydrostatic compression were shown to release increased amounts of TGF-beta activity into the medium (Klein-Nulend et al, 1995). Human osteoblast-like cells express more type I collagen mRNA and protein, and increased osteopontin and osteocalcin mRNA levels following exposure to intermittent mechanical strain (Harter et al, 1995).

## **1.3 METABOLIC AND CONGENITAL BONE DISORDERS**

### Skeletal dysplasia

The abnormal organisation of cells into tissues and its morphological results is known as dysplasia (Darby and Byers, 1994). Genetic skeletal dysplasias are disorders of pre- and post-natal skeletal growth which are characterised by some or all of the following phenotypic manifestations: short stature, bodily disproportion, deformity and functional abnormality. Included are achondroplasia (ACH), thanatophoric dysplasia (TD), and hypochondroplasia (HCH), which are fibroblastic growth factor (FGF)-related disorders of the long bones in which abnormal endochondral ossification is observed affecting the normal expansion of the cartilaginous growth plate of the long bones and base of the skull. Characteristics of all three disorders include an enlarged skull, spinal abnormalities and shortening of the limbs.

Mutations in the fibroblastic growth factor receptor (FGFR) 3 gene have been associated with all three disorders (Muenke and Schell, 1995): ACH is the most common skeletal dysplasia and is caused by an autosomal dominant mutation in the FGFR3 gene (Rousseau et al, 1994). In the heterozygote, there are minimal histological changes and a very mild phenotype; the homozygous condition usually results in death at the pre- or neo-natal stage. Hypochondroplasia has an even milder presentation. TD, or "death-bearing" dysplasia, is the most common lethal skeletal dysplasia and is inherited in an autosomal dominant fashion. Expression of the disease results in severe short-limbed dwarfism, characterised by disorganised endochondral ossification, with a lack of ordered rows of cartilage cells and chondrocytic hypertrophy. It is thought this is as a result of persistence of abnormal fetal mesenchyme transformed into severely abnormal bone and cartilage (McAlister, 1988). TD is characterised also by flattening of the vertebral bodies (platyspondyly).

### Dysostotic syndromes

A syndrome is defined as a pattern of multiple anomalies thought to be pathogenetically related and not derived from a single known or assumed prior anomaly or mechanical factor (single sequence) or from disturbances of a single development field (polytopic field defect). Included in this category are dysostoses, which is a group of developmental diseases in which individual bones or regional constellations are malformed during organogenesis (Darby and Byers, 1994).

The premature fusion of cranial sutures is known as craniosynostosis and it is a characteristic of over 100 inherited syndromes including craniofacial dysostosis (such as Crouzon's syndrome) and acrocephalosyndactyly (including Apert and Pfeiffer's syndromes). Intramembranous bone formation is adversely affected, but other phenotypic features can include fusion of the digital bones and soft tissue syndactyly. All of these diseases are characterised by one or more of the following: craniosynostosis, usually complete closure of the sutures by age 3 years, and facial, hand or feet anomalies, resulting in such clinical features as mitten hand and sock foot (Apert syndrome) and broad thumbs and toes (Pfeiffer's syndrome).

Most of these disorders are due to a single autosomal dominant mutation: craniosynostosis, Boston type, has been linked to a dominant point mutation in the MSX-2 gene leading to a substitution of a histidine for a proline in the homeodomain (Jabs et al, 1993). Crouzon, Pfeiffer, Apert and Jackson-Weiss syndromes have point mutations in the fibroblastic growth factor receptor 2 (FGFR2) gene (Reardon et al, 1994; Richman, 1995), although some Pfeiffer syndrome patients have a mutation in the same region of the FGFR1 gene (Muenke and Schell, 1995). It was demonstrated that in patients with Crouzon's disease the levels of functional FGF receptor 2 were lower than in patients with similar but non-syndromic craniosynostosis (Bresnick and Schendel, 1995). No disorder has so far been linked to mutations in the FGFR4 gene.

### Osteoporosis

Disruption of bone homeostasis can lead to a wide variety of metabolic bone diseases. Bone modelling is restricted to the young skeleton where osteoclastic resorption pits are fully refilled by osteoblasts. After development of the skeleton, the efficiency of the osteoblasts decreases over time, resulting in a net deficit of bone in the remodelling process and the overall reduction of trabecular bone in the many thousands of remodelling sites all over the skeleton (Teitelbaum, 1990).

Osteopenia was defined recently as a value of between 1 and 2.5 standard deviations below the young adult mean value; a level of bone mineral density loss equivalent to 2.5 standard deviations or greater denotes osteoporosis (World Health Organisation definition, 1994, as referred to by Kanis et al, 1996). This disease is the most common metabolic bone disorder in the Western world and projections indicate that the incidence of osteoporosis will double in the next 30 years. Currently, at least



33% of white women, who have the lowest peak adult bone mass, will have sustained a fracture in the distal forearm (Colles' fracture) and a vertebral fracture by the age of 70 years, and a hip fracture by age 90 years (Kleerekoper and Avioli, 1990). In the United States, costs incurred from treatment of this disease run into 10 billion dollars a year (Rosen, 1994); in the U.K., the current figure is greater than 750 million pounds a year (National Osteoporosis Society, 1996).

Osteoporosis manifests clinically as fracture with associated pain and deformity, occurring in the vertebrae with associated height loss and possible spinal deformity such as thoracic kyphosis (Dowager's hump). Mortality within three months following hip replacement due to osteoporotic fracture has been gauged at 20%, with permanent care required for up to half of all patients. Factors predisposing to osteoporosis include genetic elements, lifestyle components (including lack or excess of weight-bearing exercise, smoking and early natural menopause), nutritional factors and drug treatment. The incidence in men is lower than that in women due to higher peak bone mass and lower life expectancy (Jackson, 1990) and rare cases have been observed in juveniles (Norman, 1990).

Loss of bone mineral density can also occur as a result of a separate medical disorder: marrow-related disorders (including leukaemias, anaemias, lipidoses and lymphomas) and connective tissue disorders (including scurvy, osteogenesis imperfecta and homocystinuria) have been implicated in the development of osteoporosis (McKenna, 1990). It has been observed following drug treatments: pharmacological doses of glucocorticoid produces severe disease due to direct suppression of osteoblastic bone forming activity and increased parathyroid hormone secretion which stimulates osteoclastic bone resorbing activity (Hahn, 1990); treatment with heparin, anticonvulsants and methotrexate have also been found to induce bone loss. Immobility due to injury, stroke, paralysis, lack of exercise or hypogravitational conditions, such as space travel, triggers reduction in bone mass, particularly in weight-bearing bones (McKenna, 1990).

#### 1.4 THE OSTEOLASTIC CELL LINEAGE

Osteoblastic cells arise from multipotential primitive mesenchymal stem cells which, following commitment to the osteogenic lineage, differentiate progressively to preosteoblasts, mature osteoblasts, and either lining cells or osteocytes (reviewed by Aubin et al, 1993).

##### Bone marrow stromal cells

Progenitor cells are found in bone marrow stroma and can commit to several lineages: formation of muscle, fat, bone and cartilage can be induced from these multipotential cells (Grigoriadis et al, 1988). A subpopulation of bone marrow stromal cells loaded into diffusion chambers was shown to form cartilage and bone (Bab et al,

1986): the relatedness of these two lineages can be demonstrated by their ability to transdifferentiate under certain conditions (Roach, 1992; Pfeilschifter et al, 1993).

Human bone marrow contains a heterogeneous subset of bone precursor cells within which a subpopulation of proliferating osteoprogenitor cells exists: in vitro, these earlier cells produce a colony of cells which respond to treatment with calcitriol, bone morphogenetic protein-2 (BMP-2) and basic fibroblastic growth factor (FGF-2) (Long et al, 1995). The less proliferative precursors form smaller clusters of cells and have been found to be unresponsive to treatment with FGF-2 and are presumed to represent a more mature precursor (Long et al, 1995).

Bone marrow precursors are identified by their ability in vitro to produce colonies of fibroblast-like cells derived from individual clonogenic progenitors, termed colony forming unit-fibroblastic cells (CFU-F), in single-cell suspensions of bone marrow (Gronthos and Simmons, 1995). The monoclonal antibody Stro-1 binds to the cell membrane-expressed, trypsin-resistant Stro-1 antigen on a subpopulation of human bone marrow stromal cells in which all detectable CFU-F are present (Simmons and Torok-Storb, 1991). The involvement of these CFU-Fs in bone formation were shown when suspension of rat hindlimbs reduced bone growth, numbers of adherent marrow stromal cells harvested from the femur and tibia, and total population of colony forming unit-fibroblastic cells (Zhang et al, 1995).

#### Progression through proliferation and differentiation

There are fewer multipotential mesenchymal stem cells (MSCs) than tri- or bipotential cells, indicating that MSCs have a limited proliferative capability and that marginally more committed cells can proliferate more readily (Grigoriadis et al, 1990). A further expansion in population is noted with committed osteoprogenitors: preliminary osteoblast-related gene expression is maintained during proliferation while expression of post-proliferation genes is suppressed, then proliferation is down-regulated and induction of differentiation-specific genes occurs (Stein and Lian, 1993).

Genes implicated in cell growth and biosynthesis of the extracellular matrix are highly expressed during the proliferation period, including c-myc, c-fos, c-jun, histone H4, type I collagen, fibronectin and transforming growth factor-beta (TGF-beta); with development and maturation of the extracellular matrix, these are gradually suppressed and alkaline phosphatase, matrix gla protein, osteonectin and osteocalcin are expressed. In the final stage of mineralisation of the extracellular matrix, these are also down-regulated and bone sialoprotein is expressed (reviewed by Stein and Lian, 1993). In a different study, the order of expression was shown to be slightly different in the later stages, but the same idea of a cascade of gene expression was observed: expression of osteoblastic markers in rat bone marrow stromal cell cultures was shown to be variable from cell to cell but overall the temporal sequence of transcription was osteopontin, bone sialoprotein and alkaline phosphatase, then bone Gla protein, known as

osteocalcin, the latter of which coincided with a loss of marrow stromal cell proliferation (Malaval et al, 1994). In osteosarcoma-derived cells, this cascade is deregulated such that proliferation continues and depending on the cell line, certain differentiation-specific genes such as osteocalcin may be constitutively expressed (Stein and Lian, 1993).

The sequence of gene expression in vivo was examined in sheep femora by in situ hybridisation. Spindle-shaped cells were located in the first two weeks in the granulation tissue: these expressed  $\alpha 1(I)$ procollagen, alkaline phosphatase and osteopontin. As osteogenesis occurred, cuboidal cells expressing alkaline phosphatase, osteopontin and bone sialoprotein appeared and the spindle-shaped cells were no longer observed (Zhou et al, 1994).

#### Preosteoblastic phenotype

Committed osteoblastic precursors, preosteoblasts, are identified by their proximity to the osteoblast layer at the bone forming surface. They have similar morphological and ultrastructural features to osteoblasts and demonstrate alkaline phosphatase activity. Preosteoblasts display limited proliferative capacity (Nijweide et al, 1988).

#### Mature osteoblasts

In vivo, osteoblasts are cuboidal polar basophilic cells which are located directly opposed to the bone surface. These mesenchyme-derived cells are difficult to distinguish from fibroblasts based solely on morphology but can be separated due to their bone matrix production capabilities (Aubin et al, 1993). Osteoblasts are thought to have a pivotal role in the remodelling of bone as they lay down extracellular matrix which is later mineralised, and they initiate and modulate osteoclastic resorption (Nijweide et al, 1988). Osteoblasts also mediate bone resorption stimulator actions for such agents as parathyroid hormone, prostaglandin, calcitriol, interleukins 1, 6 and 11 and tumour necrosis factor alpha (Martin and Ng, 1994).

Aside from acting as a depository for secreted factors and lending strength to bone, the extracellular matrix was shown to be extremely important in the differentiation of osteoblasts: type I collagen, the major component of extracellular matrix, accelerates the maturation and differentiation of osteoblasts, and promotes mineralisation of the matrix (Lynch et al, 1995). Ascorbic acid was shown to be necessary for the ordered production of an extracellular matrix: procollagen hydroxylation is increased by ascorbic acid, which enables triple-helical conformation. This is secreted faster than non-helical collagen. Increased secretion upregulates translation and transcription of procollagen mRNAs, all of which results in a dramatic increase in matrix production. Collagen synthesized in the absence of ascorbic acid is thought to be degraded resulting in little or no extracellular matrix (Franceschi and Iyer,

1992): scurvy can induce osteoporosis as evidenced by dramatic thinning of cortical bone and extreme reduction of trabecular cross-links (McKenna, 1990).

The cascade of gene expression in the maturation of osteoblasts can be broadly defined as having three stages: in early differentiation, osteoblasts express pro  $\alpha 1(I)$  collagen, growth hormone receptor, biglycan, bone morphogenetic proteins and retinoic acid receptors; middle-stage osteoblasts express alkaline phosphatase, osteonectin, osteopontin and matrix gla protein; and late-stage osteoblasts express osteocalcin (Martin and Ng, 1994).

Maturation-dependent effects of steroid hormones on this pattern of expression was demonstrated in vitro: proliferating cells adopt a cuboidal appearance after glucocorticoid treatment, whereas continuous treatment with calcitriol results in a permanent block on differentiation; and, treatment of mature nodules with glucocorticoid produced osteoblastic cells much reduced in size, where calcitriol stimulated osteocalcin production (Pockwinse et al, 1995). This inhibition of differentiation observed with chronic (more than 48 hours) treatment of osteoblasts with calcitriol was shown in another study (Stein and Lian, 1993), whereas acute treatment (less than 48 hours' exposure) accelerated cells through proliferation and differentiation.

#### Bone lining cells and osteocytes

It is thought that osteoblasts, once they have ceased their secretory activities, can progress to bone lining cells or osteocytes. Bone lining cells are very flattened with a spindle-shaped nucleus, but contain few other organelles, consistent with the idea that osteoblastic secretory function is down-regulated in these cells (Nijweide et al, 1988). They are found on the surface of bone that is not undergoing remodelling, and are linked to other lining cells and osteocytes by gap junctions, allowing communication through which they may regulate calcium and phosphate ion fluxes, or control the growth of bone crystals (Aubin et al, 1993).

Towards the end of their secretory period, osteoblasts may become incorporated into bone matrix, where they progressively lose organelles and reduce in size, with a concomitant decrease in expression of osteoblastic cell surface markers (Nijweide et al, 1988). Initial embedment prior to expression of an osteocytic-specific marker results in the osteocytic osteoblast, or osteoid osteocyte, phenotype; as the matrix mineralises, decrease in size and parathyroid hormone (PTH) binding denotes development of the osteocyte (Rodan et al, 1988). These spider-like cells connect with each other and with lining cells via cytoplasmic extrusions (canaliculi), maintaining communication through gap junctions (Aubin et al, 1993). They maintain contact with each other, even after collagenase treatment to remove matrix, and do not proliferate in culture (Nijweide et al, 1988). They may be involved in the detection of mechanical strain and may signal bone formation or resorption accordingly (Rodan et al, 1988).

## 1.5 GENE EXPRESSION IN OSTEOLASTIC CELLS

As previously indicated, the study of gene expression in osteoblasts suggests a cascade with the expression of one gene attenuating as the next is induced. There has been some disagreement as to the exact order in which the genes studied are transcribed, but this could be due to differences in the systems used or heterogeneity of the osteoblast population. Generally, it is agreed that type I collagen is expressed prior to alkaline phosphatase and osteopontin, then bone sialoprotein and osteocalcin are expressed.

Type I collagen is vital for the mediation of a signal cascade for expression of a mature osteoblastic phenotype and for extracellular matrix mineralisation, as shown by studies in primary and passaged rat calvarial osteoblasts cultured on films of collagen as compared with those cultured on plastic (Lynch et al, 1995). Osteoblasts isolated from elderly donors accumulated more collagen than younger counterparts by inhibition of collagen degradation as evidenced by vanishingly low levels of collagenase (Koshihara and Honda, 1994).

Alkaline phosphatase is expressed on the cell surface and hydrolyses monophosphate esters at high optimum pH. Its activity increases with maturation of the osteoblastic phenotype, but is absent from the osteocyte, suggesting its use as a marker of osteoblast differentiation (reviewed by Aubin et al, 1993).

Osteopontin is an  $\alpha_v\beta_3$  integrin-binding phosphoprotein secreted by osteoblastic cells. It fosters cell attachment, influences gene expression and modulates intracellular calcium ion ( $\text{Ca}^{2+}$ ) levels (Denhardt and Guo, 1993) and has been shown to be responsive to calcitriol treatment (Noda et al, 1990). The effect on osteopontin expression is mediated in part by a ras-activated enhancer, distinct from previously described ras elements, which interacts with several unidentified proteins, one of which is associated with increased metastatic potential of the cell (Guo et al, 1995).

Bone sialoprotein (BSP) is a major constituent of mineralised tissue in vertebrates and is an acidic phosphorylated glycoprotein: sequences are highly conserved between avian and mammalian BSP, although overall homology is less than 40% (Yang et al, 1995).

Osteocalcin, also known as bone Gla protein (BGP), is a 49 kD protein which contains 3 vitamin K-dependent gamma-carboxyglutamate residues. It is secreted by osteoblasts and odontoblasts (Bradbeer et al, 1994) and is the most abundant noncollagenous protein in adult bone (Mänone et al, 1990). Gamma-carboxylation of the protein in MG-63 cells can be modified by calcitriol and retinoic acid (RA) but not by 9-cis-retinoic acid (9-RA) (Szulc and Delmas, 1996). Incompletely gamma-carboxylated bone Gla protein has been found in human bone samples, possibly due to variation in the availability of vitamin K (Cairns and Price, 1994).

Osteocalcin mRNA is expressed in chicken osteoblasts and hypertrophic chondrocytes, but not in non-hypertrophic chondrocytes, brain, heart, intestine, kidney,

liver or muscle (Neugebauer et al, 1995). Another study, in rats and humans, contradicted this: osteocalcin mRNA was detected by reverse-transcription polymerase chain reaction (RT-PCR) in liver, kidney, lung, brain, muscle and bone, although levels were 1000-fold higher in bone than in duodenal samples (Fleet and Hock, 1994).

Clues to the function of osteocalcin were suggested by the work of Ducy et al (1996). Both murine osteocalcin genes (mOG1 and mOG2) were deleted and the resulting phenotypically normal heterozygotes were mated to produce homozygous mutant mice at the expected Mendelian frequencies. These mutant mice expressed no osteocalcin and appeared normal at birth, without defects in patterning or bone formation. With time, abnormalities in bone remodelling occurred, resulting in increases in amounts of trabecular bone and mineralised bone matrix, thickness and density of cortical bone, diaphyseal width, cortical bone width and improved mechanical properties, where the failure load of the bone was increased. The number of osteoclasts was augmented, exacerbating bone resorption but this was cancelled out by the marked rise in bone formation. This was not accompanied by an increase in osteoblast number, suggesting that each osteoblast was laying down more extracellular matrix. From this, it was concluded that the normal function of osteocalcin is to constrain bone formation without affecting resorption or mineralisation, and that this negative regulation may be mediated by an as-yet-to-be identified receptor. There is however a possible role for osteocalcin in the coupling of formation to resorption (Stein and Lian, 1993).

## 1.6 REGULATION OF TRANSCRIPTION

Initiation of transcription in most prokaryotic and eukaryotic genes is usually mediated by the DNA sequence TATAAA, known as the TATA box, which is situated up to 30bp before the transcription start site. An assembly of transcription factors (TFs) and RNA polymerase II is necessary for initiation of transcription: this forms as a result of the sequential binding of TFIID and TFIIA at the TATA box, then binding of TFIIB, RNA polymerase II, and TFIIIE (Zubay, 1983a).

Other transcription factors have been discovered which are necessary for augmented levels of transcription: they can be tissue-specific and temporally regulated, and reversibly bind downstream, upstream or in the middle of the gene which they regulate. These transcription factors tend to contain at least one of the following protein structures: helix-loop-helix motif (*Drosophila* antennapedia and ultrabithorax homeotic proteins); zinc finger motif (glucocorticoid steroid receptor in vertebrates) or leucine zipper motif (mammalian c-fos and c-jun growth regulators) (Zubay, 1983b).

### Homeobox genes and homeodomain proteins

Homeotic proteins are a group of helix-loop-helix transcription factors which are responsible for the proper development of an organism by regulating specific temporal and spatial patterns of gene expression: specification of the positional information along

the anteroposterior and appendicular axes has been attributed to the homeobox (Hox) genes (St-Jacques and McMahon, 1996). Each homeotic protein contains a highly conserved 60 amino acid segment known as the homeodomain, encoded by a 180 nucleotide DNA sequence called the homeobox, present in the 3' exon of the homeotic gene. The homeodomain binds to specific DNA sequences, thus regulating gene expression (Gehring, 1994).

Homeotic proteins were first described in *Drosophila*, where they were found to regulate the development of the body plan, being expressed temporally in an order which reflects their linear arrangement on the chromosome (reviewed by Gehring, 1994). Homeotic proteins have since been found to be highly conserved throughout a wide variety of species, including mouse, chicken, frog and human (McGinnis et al, 1984). Cluster conservation is due to several constraints: co-ordinated regulation of closely located genes in a specific order; presence of enhancer sequences which upregulate the expression of a number of genes simultaneously; and, sharing cis-acting elements between adjacent genes (van der Hoeven et al, 1996).

In the embryogenesis of the *Drosophila*, co-ordinate genes, including morphogens such as the bone morphogenetic proteins, act first to produce the antero-posterior and dorso-ventral axes; then segmentation genes, of the muscle segmentation class (*msh*) amongst others, act to induce segment identity along the body plan; regionalisation genes act to specify within each segment; and finally, cell determination genes direct individual cells to their differentiated state. At all these points, various homeobox genes act to modify expression of genes (Gehring, 1994). Expression of homeobox genes may be differentiation-dependent: differential homeobox gene expression was demonstrated in human bone marrow subpopulations which had been isolated according to their cell-surface expression of CD34+ (Sauvageau et al, 1994). It is also possible that interaction of homeotic gene products may contribute to specification, determination or differentiation (White, 1994).

With so many diverse genes containing helix-loop-helix binding motifs in their promoters, it was difficult to understand how specificity of binding could arise. One mechanism could involve changes in complex DNA structure: in bone cells expressing osteocalcin, chromatin structure remodelling in the osteocalcin promoter has been demonstrated at two sites (-600 to -400 and -170 to -70, see figure 1.5) where nucleosomes are unpackaged, making the DNA sequence accessible to transcription factors and the vitamin D receptor complex (Montecino et al, 1996). Another possibility is that helix-loop-helix-containing proteins may act in partnership with other regulatory factors; where subtle differences in binding site sequence may select which Hox protein is preferred, or where protein-protein binding may alter the conformation of the Hox protein, changing the protein-DNA binding region such that a different binding site is required (reviewed by Mann and Chan, 1996).

All homeotic genes were named Hox genes until it was shown that they could be split into two groups: one which resembled the antennapedia-type complex, and another which did not. The second group contains genes which have since been renamed. One group of the non-antennapedia-type is the *Drosophila* muscle-specific homeobox (Msh) class of homeobox genes, which encode putative helix-loop-helix nuclear transcription factors. In *Drosophila*, a complex pattern of expression is observed with Msh mRNA present throughout embryogenesis and concentrated in late embryogenesis in musculature and the central nervous system (Gehring, 1994). Msh genes are expressed across phylogenetic borders: homologues are found in insects, fish, birds and mammals (De Robertis, 1994). There are at least three Msh class genes in mammals (Bürglin, 1994).

### MSX genes

Following use of the Msh gene as a probe, mammalian msh-like homeobox genes (Msx) were isolated from a mouse cDNA library and shown to be expressed during fetal development (Hill et al, 1989). In murine embryonic development, the homeodomain-containing protein Msx-2 is expressed in a range of prospective tissues including neural crest cells and their derivatives, such as the branchial and mandibular arches, and craniofacial cartilage and membranous bone (Takahashi et al, 1991), developing cranial sutures in a region of bone deposition and resorption (Jabs et al, 1993), the developing mandible and tooth (MacKenzie et al, 1992; Jowett et al, 1993) and the apical ectodermal ridge and underlying mesenchyme of the limb bud (Davidson et al, 1991). The expression pattern of this gene depends on epithelial-mesenchymal interaction (Jabs et al, 1993) and the presence of its transcript in the calvarial bones and in adjacent mesenchymal cells has led to the proposition that Msx-2 has a role in the differentiation of osteoprogenitor cells. It is thought that expression of Msx genes in limb development is under the control of growth factors secreted by the apical ectodermal ridge of the limb bud (De Robertis, 1994). These include fibroblastic growth factors (FGFs) and bone morphogenetic proteins (BMPs).

The human homologue, MSX-2, has been found to be expressed in adult human osteoblastic cells (Hodgkinson et al, 1993). As noted previously, a dominant mutation in the gene encoding the MSX-2 protein (substitution of a histidine for a proline at position 7 of the homeodomain) has been found to result in some limb deformity and cranial abnormality, leading to craniosynostosis, Boston type (Jabs et al, 1993). It is possible that this mutation affects DNA binding or protein interaction but these may not be completely ablated - some functionality may be retained (Davidson, 1995). This may explain the otherwise apparently normal phenotype.

MSX-2 expression is tightly restricted temporally and spatially and is down-regulated as development progresses (Takahashi et al, 1996). Although the pattern of MSX-2 expression is of interest, it was not until recently that studies were performed to



gain some understanding of the functions of the Msx-2 gene product. MSX-2 expression was observed in cells with relatively high proliferative potentials, including carcinoma cells and ras-transformed fibroblasts (Takahashi et al, 1996). This is due to either MSX-2 expression in the original tissues from which the tumour arose, induction as a result of malignant transformation, or as a causal event in carcinogenesis. MSX-2 overexpression in chicken myoblast culture induced transformation whereas following transfection of an antisense MSX-2 construct into ras-transformed cells, a low rate of reversion was produced (Takahashi et al, 1996). Antisense disruption of murine Msx-2 gene resulted in pronounced reduction of craniofacial bone development, severe somite and neural tube deformities and eye abnormalities (Potts and Sadler, 1997). <sup>antisense Msx-2</sup> of the mandible investigation, chick mandibular explants were cultured with antisense Msx-2 oligonucleotides and chondrogenesis in a normally cartilage-free zone of the mandible was shown (Mina et al, 1996). However, Msx-2 expression as induced by BMP-4 in the chick hindbrain precedes apoptosis (Graham et al, 1994). These studies taken together suggest therefore that Msx-2 may act to prevent differentiation, such as allowing proliferation in carcinoma cells and in transformed lines, or to block chondrogenesis, or may trigger apoptosis by supplying a contrary signal to the differentiation stimulus introduced by BMP-4.

## 1.7 REGULATION OF OSTEOCALCIN TRANSCRIPTION

As mature osteoblasts are the only extracellular matrix-secreting cells in the body to express osteocalcin, the presence of osteocalcin mRNA transcripts is taken as an endpoint of osteoblastic differentiation in this body of research. Study of the control of osteocalcin transcription has revealed an astonishingly intricate array of regulatory sequences (see figure 1.5). Elements upstream of the coding sequence, and several within it, appear to hold crucial roles in osteocalcin gene expression. Developmental, stage-specific, basal and induced expression are all tightly regulated by a range of factors, some expressed ubiquitously, others specific to bone-forming cells. Nuclear architecture and chromatin structure are also shown to have important roles.

### The osteocalcin (OC) box

The OC box is a basal regulatory element which supports tissue-specific and temporal transcription of the osteocalcin gene. This primary proximal promoter sequence (-99/-76 in relation to the osteocalcin transcription start site) consists of several overlapping regulatory sequences: there are two consensus binding sites for activator protein-1 (AP-1), one for MSX homeodomain transcription factors, a CCAAT box and a binding site for a uniquely expressed osteocalcin binding protein (OCBP). Overlapping the OC box at -102/-97 is a consensus binding sequence for E box-binding basic helix-loop-helix transcription factors.

### Activator protein-1

The activator protein-1 (AP-1) family of ubiquitously expressed proto-oncogenes, including c-fos and c-jun, affect basal transcriptional activity through the binding of a heterodimeric protein complex, such as Fos/Jun, to the consensus sequence TG/TAG/CT/CCA/C. There are three AP-1 elements in the rat osteocalcin promoter (-467/-460, -98/-92 and -88/-82) (Pike et al, 1993; Stein and Lian, 1993), the most distal present immediately 5' to the VDRE, and the second and third within the OC box.

Binding at either of the AP-1 sites in the OC box may interfere with binding at the CCAAT box, which lies between the AP-1 sites and is overlapped by them. Similar interference may occur at the VDRE. Occupancy of the three AP-1 sites by Fos/Jun heterodimers occurs during osteoblastic proliferation and suppresses osteocalcin gene expression: as proliferation is down-regulated, AP-1 activity is reduced and osteocalcin is expressed (reviewed by Stein and Lian, 1993). Inhibition of osteocalcin gene expression in proliferating osteoblasts by AP-1 occupancy of all three sites in the VDRE and the OC box simultaneously, suppressing basal and vitamin D-induced expression of osteocalcin, has been termed phenotype suppression (Lian et al, 1991).

### E boxes

Basic helix-loop-helix transcription factors can bind to the E box, the consensus sequence for which is CANNTG. One such sequence (OCE1) overlaps the distal AP-1 site within the OC box (-102/-97); a second (OCE2) is adjacent to the OSE2 element (-149/-144) (Tamura and Noda, 1994; Quarles et al, 1997). Binding at the E boxes has been shown to occur during osteoblastic differentiation: one study (Kazhdan et al, 1997) suggested binding at OCE1 was incidental, perhaps simply occurring upon release of the site by the AP-1 protein. However, treatment of fibroblastic cells with bone morphogenetic protein-2 (BMP-2) dramatically increased binding at OCE1 and osteocalcin expression was induced (Tamura and Noda, 1994), suggesting at least a supplementary role for this element in osteocalcin expression.

### MSX and OCBP binding sequences

A binding motif for the MSX family of homeodomain-containing proteins was recently found to play a role in the transcriptional control of the osteocalcin gene. Maximal levels of osteocalcin transcription were shown to be induced with an intact MSX consensus binding site, TGATTAAC, at -91/-84 (Hoffmann et al, 1994). Treatment with vitamin D increased binding at this sequence. However, this does not preclude the possibility that other factors which are not MSX-related may interact with this binding site. Indeed, the same group postulated the existence of a novel OC box binding protein (OCBP), specific to osteoblast-like cells, which binds in a sequence-specific manner to the OC box and upregulates osteocalcin transcription (Hoffmann et al, 1996).

As the OCBP and MSX consensus binding sites overlap, it may be anticipated that either a temporal constraint allows both proteins to bind independently at different

times, or competition for binding may result: Msx-2 was shown to be expressed in proliferating ROS 17/ 2.8 cells and reduced in differentiating cells, supporting the former prospect (Hoffmann et al, 1994). The discovery that, in ROS 17/ 2.8 cells, dexamethasone down-regulates Msx-2 messenger RNA levels (Towler et al, 1994) and inhibits osteocalcin expression (Aslam et al, 1995) lends support to the idea that Msx-2 specifically may be involved in the regulation of osteocalcin transcription.

#### The 3',5'-cyclic adenosine-monophosphate (cAMP) response region

The rat osteocalcin gene cAMP response region (ROCRR) at -121/-92 overlaps the OC box, encompassing an AP-1 site and the E box. Present within the ROCRR are two steroid hormone receptor binding hexameric half-sites separated by 10 nucleotides (-114/-92) which bind thyroid hormone receptor complexes (Towler and Rodan, 1995). The proximal region of the ROCRR (-99/-90) was suggested to be a necessary component of an element at -144/-138 which regulates FGF-2-mediated activation of the osteocalcin promoter (Boudreaux and Towler, 1996). This induction of expression by FGF-2 also requires an intact sequence further upstream (-678/-476, which includes the vitamin D response element) for maximal activation (Schedlich et al, 1994).

#### Glucocorticoid response element (GRE)

Glucocorticoids have been shown to down-regulate the expression of the osteoblastic phenotype indirectly, by controlling the expression or activity of osteoblastic growth factors, and directly by suppressing transcription of osteoblastic genes, including osteocalcin (Delany et al, 1994).

Immediately adjacent to the start site of transcription in the osteocalcin gene is the glucocorticoid response element (GRE). This consists of two hexameric half-sites separated by four nucleotides (-16/-1). There are two further half-elements: one within the OC box (-86/-81) and which overlaps the proximal AP-1 site and the Msx binding site, and the other at -697/-683. These elements mediate the dexamethasone-induced suppression of osteocalcin transcription with varying degrees of effect (Aslam et al, 1995).

#### The TATA and CCAAT boxes

Contiguous with the GRE is the TATA box (-31/-25). This sequence is universally conserved and serves to direct the RNA polymerase II transcriptional apparatus to begin transcription approximately 30 nucleotides downstream (Darnell et al, 1986).

In vivo transcription usually requires one or two elements between -110 and -40. Within the OC box is situated a CCAAT box (-92/-88), an element which has been shown to be necessary for the basal transcription of several eukaryotic genes, including globins (Darnell et al, 1986). It is possible that this element is inaccessible during

osteoblastic proliferation due to the occupancy of the AP-1 sites surrounding it, thereby preventing transcription.

#### The vitamin D response element (VDRE)

The vitamin D response element (VDRE) consists of two hexameric half-sites separated by three nucleotides. Its consensus sequence is A/GGGNNA/C AT/CG A/GGGNNA/C (Demay et al, 1990). The VDREs in the human and rat osteocalcin promoters differ by three nucleotides (underlined) (Pike et al, 1993):

Human	GGGTGA acg GGGGCA
Rat	GGGTGA atg <u>AGGACA</u>

Vitamin D-mediated upregulation of osteocalcin transcription is dependent upon ligand and receptor occupancy of the VDRE (Breen et al, 1994b): binding of the ligand (calcitriol) to the vitamin D receptor (VDR) alters its conformation, so facilitating binding of an accessory factor, usually the 9 cis-retinoic acid receptor (RXR) (Haussler et al, 1995), although the VDR can synergise with itself and several other transcription factors (Liu and Freedman, 1994) with varying affinities (Schrader et al, 1994). Treatment with 9 cis-retinoic acid reduces binding at the VDRE, probably because RXR homodimers are formed instead of VDR:RXR heterodimers, thus inhibiting vitamin D-mediated osteocalcin transcription (Haussler et al, 1995).

The VDR gene is differentially spliced to yield three unique mRNA species (Miyamoto et al, 1997). This, and varying conformations of VDR:RXR heterodimers may allow recognition of different VDREs (Staal et al, 1996a). The VDR protein consists of an N-terminal zinc finger DNA binding domain, a hinge section, and a C-terminal hormone binding domain, which contains two regions allowing heterodimerisation with an RXR (Haussler et al, 1995). To permit binding of the VDR:RXR to the VDRE and to allow transactivation, the VDR must be phosphorylated (Desai et al, 1995).

Competition for binding sites at the VDRE is an important factor in the regulation of osteocalcin expression. AP-1 counteracts binding of the VDR complex to the AP-1/ VDRE site in the osteocalcin promoter (Jääskeläinen et al, 1994): vitamin D-mediated stimulation of osteocalcin expression is accompanied by weak binding of the AP-1 heterodimer. The ubiquitously expressed YY1 factor antagonises VDR:RXR activation of osteocalcin expression in two ways: YY1 directly competes for binding at the VDRE, and YY1 can also bind to TFIIB, thereby inhibiting transcription (Guo et al, 1997). Calreticulin, a calcium binding protein, inhibits DNA binding and transcriptional activity of a range of steroid nuclear hormone receptors, including VDR, by binding to the DNA binding domain of the receptor (Wheeler et al, 1995). Transcription of calreticulin was shown to decline with osteoblastic differentiation so alleviating transcriptional inhibition (St-Arnaud et al, 1995).

Transforming growth factor-beta (TGF-beta) can interfere with the effects of vitamin D on osteocalcin transcription by mechanisms other than interference with VDR:RXR binding to a VDRE oligonucleotide or than by the serine/threonine pathway (Pirskanen et al, 1994). Other studies have shown that increased AP-1 activity was observed and reduced VDR:RXR binding to the genomic VDRE was demonstrated following TGF-beta treatment (Staal et al, 1996b; Banerjee et al, 1996a). The latter investigation showed the necessity of a region at -146/-139, which binds Fra-2, a factor hyperphosphorylated by TGF-beta, for mediation of TGF-beta-induced osteocalcin repression at the proximal basal promoter.

Critical for maximal induction of calcitriol-mediated transactivation of osteocalcin expression is an intact sequence immediately downstream of the VDRE at -420/-414, which is incapable of binding the VDR:RXR complex alone (Sneddon et al, 1997). Also, vitamin D modulates osteocalcin expression by post-transcriptional mechanisms through extension of the half-life of osteocalcin transcripts (Mosavin and Mellon, 1996).

#### Osteocalcin-specific elements (OSEs)

Two cis-acting elements were discovered near to the OC box: both were shown to increase the activity of the osteocalcin promoter in osteoblastic cells only. OSE1 (-64/-47) bound a factor present in nonmineralising and mineralising osteoblast-like cells; OSE2 (-146/-132) was active only in mineralising osteoblasts (Ducy and Karsenty, 1995). This latter element was shown to contain a consensus binding site for the runt homology domain (Merriman et al, 1995). An osteoblast-specific factor (OSF) binds to OSE2 at -135/-130 and was shown to be related to the polyoma enhancer binding protein 2/ acute myeloid leukaemia/ core binding factor-alpha (PEBP2/AML/CBFA) DNA binding protein family, the human homologue of the *Drosophila* runt family of transcription factors (Geoffroy et al, 1995; Ducy et al, 1996; Zeng et al, 1997). The bone-related OSF (CBFA1 or AML-3) protein transcriptionally activates the osteocalcin gene in rat osteoblasts (Banerjee et al, 1996b) but interestingly in mice, this activation is abolished by calcitriol, which interferes with the binding of OSF to the OSE2 (Zhang et al, 1997). The mechanism of ascorbic acid-induced osteocalcin expression was suggested to involve the OSE2 as binding of CBFA1 to the OSE2 was increased by ascorbic acid treatment (Xiao et al, 1997).

In an elegant series of experiments by Ducy et al (1997), gene expression in CBFA1 <sup>-/-</sup> mice was examined. Alkaline phosphatase, osteopontin and osteocalcin mRNA levels were extremely low;  $\alpha$ 1(I)collagen expression was abolished. MC3T3-E1 cells, which model undifferentiated osteoblastic cells, and C3H10T1/2 cells, pluripotent fibroblasts not committed to the osteoblast lineage, do not express genes characteristic of the osteoblast phenotype. They were transfected with a construct containing CBFA1, following which expression of bone sialoprotein, osteocalcin and  $\alpha$ 1(I)collagen was

shown. Antisense CBFA1 introduced to ROS 17/ 2.8 cells, which model mature osteoblasts and express genes of the osteoblast lineage, resulted in abolition of  $\alpha 1(I)$  collagen and osteocalcin expression and dramatic suppression of osteopontin expression. Taken together, these data suggest that CBFA1 is essential for the proper differentiation of cells of the osteoblastic lineage and that CBFA1 must be constitutively expressed in mature osteoblasts.

Other unidentified sequences upstream of the osteocalcin gene were shown to be required in osteocalcin transcription, possibly to enable formation of a more complex nuclear structure (Frenkel et al, 1996). Promoter activity has also been shown to be affected in vivo by age and gender, with females exhibiting greater activity than males, and by the site at which bone was examined: variation was reduced upon ex vivo culturing of these cells, leading to the suggestion that a factor not present in culture and changing with age and gender may be responsible for the differences observed (Frenkel et al, 1997).

### Silencers

Downstream effects have been noted with several elements within the first exon in the osteocalcin gene contributing towards the repression of transcription until developmentally appropriate stages. The region +24/+151 also contains an antisilencer (+40/+48) whose effect is swamped by the inhibition engendered through the whole region (Frenkel et al, 1994). The +29/+35 sequence is critical for the silencing function: repression is mediated by the binding at +17/+36 of a nuclear factor present in proliferating osteoblasts but absent in post-proliferative osteoblasts, expression of this factor being down-regulated upon mineralisation of the extracellular matrix (Li et al, 1995).

Protein: DNA binding was observed at an element (TTTCTTT) in the first intron which results in suppression of osteocalcin transcription in a range of osteoblast-like cells (Goto et al, 1996).

### Nuclear architecture

When expressing osteocalcin, ROS 17/ 2.8 cells exhibit DNase I hypersensitivity at two sites in the osteocalcin promoter; -590/-390, which encompasses the VDRE, and -170/-70, which includes the ROCRR, OC box and the CBFA1 binding sites: non-hypersensitivity is registered in cells which do not express osteocalcin (Montecino et al, 1994). Nucleosomal structure also altered to allow expression of the mature osteoblastic phenotype (Montecino et al, 1996).

Contained within the VDRE are two binding sites for nuclear matrix proteins (NMPs) at -452/-449 and -441/-435, designated NMP-1 and NMP-2 binding sites respectively (Stein and Lian, 1993); NMP-2 also binds at -605/-599. A component of

NMP-2 was shown to be a member of the runt domain transcription factor family which associates increasingly with the nuclear matrix during differentiation (Merriman et al, 1995; Lindenmuth et al, 1997). This supports the notion of involvement of the nuclear matrix in the expression of osteoblast-specific genes.

Thus, the organisation of gene regulatory elements, subtleties in recognition between similar sequences by similar factors, chromatin structure, nucleosome organisation and interactions between genes and nuclear matrix all contribute to the increasingly complex array of mechanisms involved in the transcriptional regulation of the osteocalcin gene.

## 1.8 FACTORS AFFECTING BONE FORMATION

A large group of related mediators known as the transforming growth factor-beta (TGF- $\beta$ ) superfamily consists of several subfamilies including the bone morphogenetic proteins (BMPs) (see figure 1.6, after Massagué et al, 1996). These, and calcitriol, have significant effects on bone formation.

### TGF-beta and osteogenesis

TGF-beta is a 25,000 Dalton protein composed of two identical polypeptides linked by disulphide bonds. There are multiple TGF-beta isoforms which all share similar properties: increased DNA and type I collagen synthesis, the latter as a result of increased numbers of collagen-producing cells (Centrella et al, 1986). TGF-betas are secreted to the extracellular matrix as latent high molecular weight complexes consisting of the amino terminal remnant of the latency-associated protein and which may also include TGF-beta binding protein. They are activated following proteolytic cleavage, for example during bone resorption (Keski-Oja et al, 1996).

TGF-beta upregulates the proliferation of a wide range of cells including fibroblasts, vascular smooth muscle cells, articular chondrocytes (Okragly et al, 1994; Postlethwaite et al, 1994) and myogenic cells (Katagiri et al, 1994; Yamaguchi et al, 1995). Cyclins and cyclin dependent kinases are cell-cycle regulatory factors which are expressed during proliferation, but are not restricted to this phase only: in normal rat calvarial osteoblasts, several cyclins persist in confluent cultures, suggesting a role for these molecules in osteoblastic differentiation. TGF-beta treatment during proliferation attenuates the enhanced expression of these cyclins. In confluent tumour-derived cells (ROS 17/2.8), the augmented cyclin levels are not observed postproliferatively, consistent with the notion of ablation of growth control and differentiation regulatory mechanisms (Smith et al, 1995).

The proliferative effects of TGF-beta are linked to a negative effect on differentiation. Myotube formation is blocked following TGF-beta treatment in myogenic cells but they are not induced to become osteogenic (Katagiri et al, 1994; Yamaguchi, 1995). Osteoblastic cells cultured constantly in the presence of TGF-beta 1

are irreversibly impeded from differentiating into mature osteoblasts (Breen et al, 1994a).

TGF-beta is required for production of a mature crosslinked collagenous extracellular matrix (Feres-Filho et al, 1995) as it stimulates matrix synthesis by increasing collagen and hyaluronic acid expression (Postlethwaite et al, 1994). Upon secretion to the bone matrix, TGF-beta 1 binds to decorin, a bone matrix proteoglycan, and when bound the inhibitory effect of TGF-beta on cell proliferation is enhanced, although decorin alone has no effect. It is thought this synergistic effect is mediated through enhanced binding of TGF-beta to its receptors, types I and II and betaglycan, resulting in increased bioactivity following resorption when TGF-beta and decorin are both released from the matrix (Takeuchi et al, 1994). Neutralising antibodies to TGF-betas 1, 2 and 3 were applied to extracellular matrix derived from the rat osteoblastic ROB-C26 cell line. Cells plated onto this extracellular matrix were then found to produce no osteoblastic markers of expression, suggesting that TGF-beta secreted into the extracellular matrix has an important role in the development of the osteoblastic phenotype (Kirk and Kahn, 1995). Following dexamethasone treatment of a human osteoblastic cell line, SV-HFO, expression of the tetranectin gene is upregulated and mineralisation occurs. TGF-beta treatment suppresses expression of tetranectin (Iba et al, 1995) and alkaline phosphatase in the early phase of mineralisation, indicating phase-dependent effects of TGF-beta treatment (Iba et al, 1996).

Tissue inhibitor of metalloproteinases (TIMPs) proteins are localised to the extracellular matrix: in the mouse, TIMP-1 and TIMP-3 are inducible by TGF-beta 1 (Leco et al, 1994). In fibroblasts and osteoblasts, TGF-beta 1 diminishes the expression of matrix metalloproteinases (MMPs) and augments the transcription of TIMP-1 (Overall, 1995). Ecto-nucleoside triphosphate pyrophosphatase (ENTP) is a potential source of inorganic phosphate, which may be involved in mineralisation. TGF-betas 1 and 2 have been found to induce bone-derived, but not tooth-derived, ENTP activity in a dose-dependent manner up to 10 ng/ ml and also to diminish alkaline phosphatase (AP) activity over the same dose range. If AP hydrolyses extracellular pyrophosphate in bone, then TGF-beta modulation of the activities of both these proteins results in an increase in extracellular levels of pyrophosphate and may therefore affect mineralisation (Oyajobi et al, 1994).

The expression of an important osteoblastic gap junction protein, connexin-43, normally expressed with an increase in cell density, was promoted by treatment with TGF-beta but was not influenced by calcitriol. Similar effects were noted in relation to gap junctional intercellular communication, suggesting that accretion of intercellular communication between osteoblastic cells by TGF-beta might maintain bone as an organised tissue in vivo (Chiba et al, 1994). In a variety of osteoblast-like cells, it was observed that bone matrix proteoglycan synthesis was stimulated by TGF-beta treatment only in those cell types which expressed types I and II TGF-beta receptors.



Therefore, changes in the cell-surface TGF-beta receptors may account for the variations in response to treatment with TGF-beta and subsequent expression of biglycan and decorin (Takeuchi et al, 1995).

TGF-beta has also been found to induce cartilage formation (Noda and Camilliere, 1989). TGF-betas 1, 2 and 3 are expressed in cultured chick limb mesenchymal cells at the initiation of and during cartilage differentiation (Roark and Greer, 1994). TGF-beta-1 fosters smooth muscle differentiation in mouse neural crest stem cells (Shah et al, 1996) and chondrogenesis in high density C3H/10T<sub>1/2</sub> cells (Denker et al, 1995) and rat calvarial cells, but blocks the formation of hypertrophic chondrocytes (Moses and Serra, 1996). Chick growth plate chondrocytes treated with TGF-beta showed increased DNA synthesis after 24 hours and inhibited alkaline phosphatase activity. A dose-dependent increase in chondrocyte proliferation was seen following treatment with TGF-beta (Guerne et al, 1994). The expression of osteonectin in chondrocytes is upregulated by TGF-beta at transcriptional and translational levels (Nakamura et al, 1996).

Bone is the largest reservoir of TGF-beta in the body. The effects of TGF-beta on osteoblastic cells have been extensively reviewed (Centrella et al, 1989; Bonewald and Mundy, 1989; Kim and Ballock, 1993). Transforming growth factors  $\beta$ 1 and  $\beta$ 2 (TGF-betas 1 and 2) are present in large quantities in the bone matrix (Seyedin et al, 1985) and have been found to stimulate the proliferation, chemotaxis and differentiated function of cells of the osteoblast lineage (Mundy, 1995) and to stimulate bone formation in vivo (Oue et al, 1994).

Genetic effects of TGF-beta in osteoblastic cells have been examined: TGF-beta 1 application to the rat preosteoblast cell line UMR 201 induced matrix gla protein (MGP) and promoted the steady state levels of osteonectin (ON) and pro-alpha 1 (I) collagen mRNA. Osteopontin (OP) expression was reduced (Zhou et al, 1993). Treatment of subcultures of fetal rat calvarial osteoblasts with TGF-beta inhibited bone nodule formation and the expression of genes associated with osteoblast differentiation was diminished: type I collagen, alkaline phosphatase, osteopontin, BMP-2 and osteocalcin levels were down-regulated. This suggests that TGF-beta can affect less differentiated cells in the osteoblastic lineage (Harris et al, 1994a).

TGF-beta augments transcription, ligand binding and parathyroid hormone/parathyroid hormone related protein (PTH/PTHrP) receptor signalling in ROS 17/2.8 cells (McCauley et al, 1994). Also, in MC3T3-E1 cells, TGF-beta was shown to inhibit PTH/PTHrP receptor expression and osteocalcin expression persistently in less differentiated osteoblasts - similar but not persistent effects were noted in more mature cells, leading to the suggestion that TGF-beta inhibits osteoblastic maturation in earlier cells (McCauley et al, 1995).

TGF-beta influences the expression of transcription factors: a novel TGF-beta-inducible early gene (TIEG) and putative transcription factor expressed only in

osteoblasts and skeletal muscle tissue may be important in osteoblast intracellular signalling (Subramaniam et al, 1995). In MC3T3-E1 cells, TGF-beta exaggerates the expression of retinoic acid and retinoid X receptors (RAR and RXR respectively): RAR-alpha, RAR gamma, RXR alpha genes are transcriptionally activated; RAR beta, RXR beta and RXR gamma are not. Transcriptional activation is inhibited by addition of antisense c-fos and c-jun, implying a role for AP-1, a transcriptional activation complex composed of Fos and Jun proteins (Chen et al, 1996).

TGF-beta has biphasic effects on osteoblast proliferation and activity which appear to be differentiation-dependent. Differences in the quantities of type I and II receptors on the cell surface between fibroblast bone-derived cells and highly differentiated osteosarcoma cultures, respectively reflecting early and late stage osteoblast phenotype, point to the regulation of receptor expression as a mechanism for this effect (Centrella et al, 1995; Moses and Serra, 1996). Type II receptor expression was found to decrease relative to that of the type I receptor with increased osteoblastic activity.

TGF-beta was seen to regulate the expression of its own receptors: in MC3T3-E1 cells treated with TGF-beta, diminished levels of TGF-beta receptors were expressed on the cell surface. The mechanism was thought to be related to the interaction of collagen matrix induced by TGF-beta and  $\alpha 2\beta 1$  integrin expressed on the osteoblast surface (Takeuchi et al, 1996). The TGF-beta type-III receptor, betaglycan, is expressed prodigiously in osteoblastic cells and is a cell surface heparan/chondroitin sulphate proteoglycan. Binding of TGF-beta to betaglycan is ameliorated by dexamethasone (dex) treatment: dex was shown to augment de novo transcription of betaglycan, but not of TGF-beta type II receptor (Nakayama et al, 1994). Cell surface TGF-beta receptors have a rapid turnover rate; inhibition of de novo transcription resulted in a slower loss of cell surface receptor than protein synthesis inhibition (Centrella et al, 1996).

Intracellular signalling by members of the TGF-beta superfamily remains to be fully elucidated. However, a putative model of transduction has been put forward (Derynck and Zhang, 1996). TGFs signal by binding first to a type II serine/threonine kinase receptor which then forms a heteromeric receptor complex by binding to and phosphorylating a type I serine/threonine kinase receptor (Dewulf et al, 1995). An intracellularly associated Smad (vertebrate homologue of the mothers against decapentaplegic protein first noted in *Drosophila* and which has homology with the human tumour suppressor gene DPC4) is subsequently phosphorylated and dissociates, binding to and synergizing with another Smad. This complex is translocated to the nucleus where Smads function as transcriptional activators: Mad 3 has been shown to act as a mediator linking TGF-beta/ activin receptors to transcriptional regulation (Derynck and Zhang, 1996).

Targets of this transcriptional activation as mediated by Smads may include cyclin-dependent kinase inhibitor p15 (p15), expression of which blocks cdk4 and cdk6,

and leads to cell arrest in G1 (Moses and Serra, 1996; Massagué, 1996), and dwarfins, which are phosphorylated in a dose- and time-dependent manner following treatment with TGF-beta. BMP-2 treatment also resulted in phosphorylation of one dwarfin, suggesting that dwarfins may act as mediators for the TGF-beta superfamily. It was postulated that Smads are tumour suppressor genes as growth arrest is blocked in tumour cells which are resistant to TGF treatment (Alexandrow and Moses, 1995) and transient overexpression of two murine dwarfins, which are downstream of Smads, caused growth arrest (Yingling et al, 1996).

### Bone morphogenetic proteins and bone formation

A morphogen is a protein which induces morphological change depending on its concentration (Gehring, 1994). Bone morphogenetic proteins BMP-1 to BMP-7 were isolated from bovine bone matrix and their genes cloned from human cDNA libraries (Wozney et al, 1988), and based on their homology, four sub-groups were found: BMP-1 alone bearing little homology to any of the other BMPs; BMP-2 and BMP-4, the human equivalent of the *Drosophila dpp* gene; BMP-3 alone; BMP-5, BMP-6 and BMP-7. Six further members of the BMP family have since been cloned and sequenced (Dube and Celeste, 1995; Celeste et al, 1995). BMPs are expressed in a wide variety of tissue other than bone and are therefore assumed to have a fundamental role in the regulation of the early development of the embryo. They are thought to be secreted and then sequestered in or on the bone matrix (Mundy, 1995).

BMPs stimulate osteoblast differentiation by enhancing the expression of alkaline phosphatase and osteocalcin, and also induce the formation of mineralised bone nodules in vitro under the appropriate culture conditions. They have proven to be useful in healing bone defects: craniosynostotic defects were treated with a combination of lyophilised cartilage and BMP, which appeared to accelerate the ossifying and calcifying effects of the lyocartilage (Sailer and Kolb, 1994); and, following treatment of craniotomy defects in rats with rhBMP-2, it was shown that BMP-2 promotes the regeneration of osseous tissue (Marden et al, 1994).

### Individual BMPs

BMP-1 is co-purified with other BMPs from active demineralised bone matrix, suggesting interaction with these other proteins (Shore et al, 1995). It is not a member of the TGF-beta superfamily but is similar to *Drosophila* tolloid protein and suggested to be a member of a family of astacin proteases which is involved in pattern formation in insect development (Shimell et al, 1991; Takahara et al, 1994). BMP-1 in mammals has been identified as procollagen C-proteinase: the carboxy-termini of procollagens I, II and III are cleaved by this protease to produce components of the extracellular matrix (Kessler et al, 1996).

The other BMPs have osteogenic effects: osteogenin (BMP-3) has been shown to have regenerative properties when applied to bone defects created in rats and baboons (Marden et al, 1993; Ripamonti et al, 1993b; Stevenson, et al, 1994). A spontaneous mutation in the short ear locus of mice was shown to result in the deformation of bone and the development of soft tissues (Kingsley et al, 1992). King et al (1994) showed that this was due to a nonsense mutation in the gene for BMP-5 and that the pattern of expression of this gene spatially resembled that of forming skeletal elements. An implied role for BMP-6 is in the terminal differentiation of chondrocytes: BMP-6 expression was transiently increased then down-regulated, following which type II collagen was decreased and finally collagen type X was stimulated. Simultaneously, cell size progressively increased (Carey and Liu, 1995).

Comparison of the sequences for murine and human BMP-2 revealed that structuring of the gene was very different between the two species although the amino acid product is over 90% homologous (Feng et al, 1994). In the same study it was shown also that BMP-2 and BMP-4 have great similarity at the genetic level only in the last exon and in regions of the 5' flanking sequence, suggesting conservation of regulatory elements. In the carboxyterminal region, BMP-2 is 75% homologous to the *Drosophila* dpp protein, which is a potent dorsalizing factor in the *Drosophila* embryo (Rusch and Levine, 1996), and can induce endochondral bone formation in the rat subcutaneous bone induction model (Sampath et al, 1993).

A clone spanning the entire BMP-4 gene was analysed, showing that two transcriptional start sites were present (5' to exons I and II) and that different variants could be expressed as a result. No TATA box was indicated within 300bp of either transcriptional start site but two Sp1 sites were found (Kurihara et al, 1993). Creation of null BMP-4 mutants in mice resulted in death of murine embryos at 6.5-9.5 days post-coitum (dpc): some do reach the somite stage or beyond but are developmentally delayed with many posterior structures truncated or disorganised. These embryos may have been partially rescued from the effects of the BMP-4 mutation by the presence of maternally-derived transcripts of BMP-4 (Winnier et al, 1995). BMP-4 is expressed in the presumptive dental epithelium at the inception of tooth development. This induces expression of BMP-4, and Msx-1 and Msx-2 (homeodomain-containing transcription factors) in the mesenchyme in a paracrine manner (Vainio et al, 1993). It is thought that this is mediated by the binding to syndecan-1, a cell surface heparan sulphate proteoglycan expressed in the dental mesenchyme, of tenascin, a matrix glycoprotein: growth factors expressed in the dental epithelium, such as BMP-2 and -4, then bind to syndecan-1 and induce the expression of Msx-1 and Msx-2 in the dental mesenchyme (Thesleff et al, 1996). Alkaline phosphatase activity is upregulated in preodontoblasts by treatment with BMPs, and expression of osteocalcin and type I collagen is induced by BMP-2 and BMP-4 respectively (Nakashima et al, 1994).

BMP-7 mRNA is present in the heart, kidney, limb bud, tooth and intestine of human fetuses (Helder et al, 1995). BMP-7 or osteogenic protein-1 (OP-1) is structurally related to TGF-beta and is 70% homologous to the Drosophila 60A protein in the carboxyterminal region - 60A protein induced endochondral bone formation in the rat subcutaneous bone induction model (Sampath et al, 1993). Ectopic expression of 60A in Drosophila embryos lead to defects in adult structures or lethality during metamorphosis (Staehling-Hampton et al, 1994).

BMP-7 was found to be expressed in the hypertrophic chondrocytes adjacent to and at the ends of the metaphyseal vessels of the chick (Houston et al, 1994). BMP-7 has been found to induce new bone formation subcutaneously in rats when implanted with a suitable carrier, and can repair defects: treatment of canine long-bone defects with BMP-7 resulted in regeneration of tissue to 67-92% of control levels (Cook et al, 1994). It is not a fibroblastic mitogen nor does it stimulate matrix synthesis (Postlethwaite et al, 1994). Primary rat calvarial cells treated in vitro with BMP-7 prior to confluence became chondroblastic, whereas when treated at a more confluent stage, cells of an osteoblastic phenotype were found. Co-treatment with TGF-beta-1 resulted in cancellation of chondrogenesis (Asahina et al, 1993). BMP-7 reduces proliferation and stimulates development of the osteoblastic phenotype: it was shown to inhibit the synthesis of DNA in ROS 17/ 2.8 cells in a dose-dependent manner (Maliakal et al, 1994) and simultaneously increase the levels of type I collagen mRNA, and the levels of osteocalcin and alkaline phosphatase activity and mRNA. The proliferative and differentiative effects of BMP-7 on human bone cells were shown to be mediated at least in part by BMP-specific receptors (Malpe et al, 1994).

#### BMP-2 and BMP-4 in development

The role of BMPs in the development of organisms has been studied in a range of species. In the developing mouse, BMP-4 mRNA is expressed early and is restricted solely to the facial epithelium; BMP-2 is expressed later in the neural crest mesenchyme, where *Msx-2* transcripts are found, and in the developing mouth (Bennett et al, 1995). BMP-2 and BMP-4 transcripts were found in the developing facial primordia of the chick: they were localised to the epithelium initially, then in the mesenchyme, with some overlap in the expression patterns of the genes (Francis-West et al, 1994).

BMP-2 and BMP-4 transcripts are stored maternally in the *Xenopus* egg, and BMP-2 is expressed at the gastrulation stage throughout the ectoderm where BMP-4 transcription is restricted to the ventrolateral marginal zone (Hemmati-Brivanlou and Thomsen, 1995). BMP-4 has a specific ventralising function which is conserved in insects and mammals: in *Xenopus* gastrula, BMP-4 is expressed in regions complementary to the goosecoid gene (*gsc*) where gain and loss of function experiments performed using antisense RNA injections show that BMP-4 ventralises dorsalised

mesoderm, as does antisense *gsc*, and vice versa (Steinbeisser et al, 1995). Ectopic ectodermal *dpp* expression during gastrulation of the *Drosophila* embryo resulted in respecification of dorsoventral patterning and in cells where 60A was also expressed no inhibition of *dpp* function was shown (Staehling-Hampton et al, 1994). The *Drosophila* short gastrulation gene *sog*, an antagonist of *dpp*, was shown to have similar effects in *Xenopus* embryos as mutated BMP receptors, that is, overexpression of *sog* resulted in dorsalisation of the embryo, also allowing partial rescue of ventralised embryos. These suggest that *sog* acts in part by opposing BMP-4 signalling (Schmidt et al, 1995). Another BMP-4 inhibitor, chicken ovalbumin upstream transcription factor-1 (COUP-TF1) was found to bind to response regions in the BMP-4 promoter, abrogating its activity (Feng et al, 1995).

BMP-2 and BMP-4 may play a part in the specification of the limb/wing bud: BMP-2 is expressed in the apical ectodermal ridge (AER) and abolishes limb outgrowth, as is FGF-4, a potent instigator of mesenchymal proliferation. It seems likely therefore that limb outgrowth is regulated by the ratio between the inhibitory and stimulatory signals of BMP-2 and FGF-4 respectively (Niswander and Martin, 1993). BMP-2 expression in anterior cells of the chick limb bud is activated by retinoic acid, but BMP-2 does not have any apparent polarising activity. BMP-4 has a similar expression pattern in the wing bud. It was observed that *Hoxd-13* and BMP-2 expression appears to be activated simultaneously upon treatment with retinoic acid (Francis et al, 1994).

#### BMP-2 and BMP-4 in bone formation

BMPs have been found to induce endochondral bone formation by causing chemotactic events (infiltration of cartilage and bone cell precursors), proliferation and differentiation of precursors into chondrocytes, induction of vascularisation, maturation of chondrocytes and differentiation of cells into osteoblasts (Reddi, 1981). Recombinant or endogenous bone morphogenetic proteins induce de novo bone formation when adsorbed onto biomaterials, such as a collagen carrier (Aaboe et al, 1995; Kusumoto et al, 1995). A gel consisting of reconstituted basement membrane, laminin or collagen without addition of other components was shown to increase bone repair at fracture sites in rats 6- to 12-fold when compared with untreated fractures left to heal without intervention (Sweeney et al, 1995).

Ectopic bone formation was induced by BMP treatment of rats: homologues of Hox cluster genes and two *Msx* genes were found to be expressed in the BMP-implanted tissue (Iimura et al, 1994b). BMPs 2, 4 and 6 were shown to induce bone nodule formation in cultures of rat osteoprogenitor cells, suggesting that these are targets for the action of BMPs in bone induction, with BMP-6 affecting an earlier stage progenitor than BMP-2 or -4 (Hughes et al, 1995). BMPs 1, 2, 4 and 6 were shown to be expressed by rat calvarial osteoblasts coincident with expression of alkaline

phosphatase, osteopontin and osteocalcin, and prior to formation of mineralised bone nodules (Harris et al, 1994b). BMP was implanted in mice: type I collagen levels increased from day 3 to 7 and on day 17, increased expression correlated with the incidence of osteoblasts. Type II collagen mRNA expression was augmented from day 7 on the appearance of chondroblasts, but suppressed by day 17 when degenerative and hypertrophic chondrocytes were in attendance. Alkaline phosphatase expression was dramatically enhanced from day 7 to day 11, at which point mineral deposition began (Sakano et al, 1993). Similarly, rat abdominal muscle was implanted with a diffusion chamber containing rabbit BMP. After two weeks, chondrogenesis had occurred outside the chamber and after four weeks this was replaced with bone (Ono et al, 1994). Activation of c-fos transcription by BMP-2 and BMP-3 in the late phase may mediate the transcription of the genes which mark differentiation of osteoblasts (Ohta et al, 1992).

With age, bone marrow stromal cells differentiate along the adipogenic pathway (Glowacki, 1995). Upon treatment of a bone marrow stromal cell line, BMS2, with BMP-2, adipogenesis was suppressed and alkaline phosphatase induced at concentrations of BMP-2 in the range 50-500 ng/ ml. It was also found that the cells constitutively expressed BMP-4 mRNA which was suppressed following treatment of the cells with adipogenic agonists, suggesting that BMPs act as adipogenic antagonists (Gimble et al, 1995).

Endochondral ossification is induced by BMP-2 by stimulated proliferation and accelerated differentiation of pre-osteoblastic cells (Ghosh-Choudhury et al, 1996) and mesenchymal cells into chondroblasts and osteoblasts, elevating the production of cartilage and matrix, and promoting the terminal differentiation of osteoclasts (Rosen et al, 1994; Zheng et al, 1994). This accelerated osteogenesis is mediated by TGF-beta receptors, the expression of which is altered by BMP-2, therefore modulating the effects of TGF-beta and augmenting the osteoblastic function of the cells (Centrella et al, 1995). Chick limb mesenchymal cells treated with BMP-2 produced more extracellular matrix - this effect was amplified in condensed or differentiated cells (Roark and Greer, 1994). BMP-2 treatment of the murine mesenchymal stem cell line C3H10T1/2 caused dose-dependent differentiation of these multipotent cells into adipocytes at low concentrations, and chondrocytes and osteoblasts at high concentrations: these phenotypes remained constant when the agent was removed (Wang et al, 1993). As well as precipitating differentiation of mesenchymal cells, BMP-2 exhibits transdifferentiation abilities with more committed cells: prechondroblasts (Rosen et al, 1994) and myoblasts (Katagiri et al, 1994) were converted into osteoblastic cells by treatment with BMP-2.

These differentiation-stimulating effects of BMP-2 are mediated by enhancement of osteoblast-specific gene expression. BMP-2 induced expression of insulin-like growth factors (Canalis and Gabbitas, 1994) and TGF-beta 1 in osteoblast-

like cells (Zheng et al, 1994). Importantly, BMP-2 mRNA expression was found to be autoregulated (Ghosh-Choudhury et al, 1996). BMP-2 treatment of osteoblastic cells results in decreased TGF-beta type II receptor expression, but type I receptor levels remained constant so it would appear that the ratio between the receptor types is important in the development of a more differentiated phenotype (Centrella et al, 1995).

BMP-4 is expressed by osteoprogenitor cells and not by differentiated osteoblasts (Nakase et al, 1994). However, ectopic introduction of BMP-4 resulted in bone formation and the sequential expression of osteonectin, osteopontin and osteocalcin, localised respectively to fibroblast-like cells; hypertrophic chondrocytes and middle stage osteoblasts; and, middle to late stage osteoblasts and a subset of osteocytes (Hirota et al, 1994).

BMP-4 treatment of a rat preosteoblast cell line (UMR 201) resulted in no effect on matrix gla protein (MGP) or osteonectin (ON) mRNA expression, an augmentation of pro-alpha1(I) collagen and a reduction in osteopontin (OP) mRNA levels. Following co-treatment with retinoic acid (RA), MGP mRNA expression was enhanced, a further increase in pro-alpha 1 (I) collagen levels was observed, although the transcription rate of this gene was unaffected, and the retinoic acid-induced increase in the OP transcription rate was not reduced (Zhou et al, 1993).

In their mature forms, BMPs can form dimers, therefore heterodimers may affect bone formation by increasing or decreasing the effects of the homodimers: BMP-2 and BMP-7 have been found to be co-localised in the zone of polarising activity, the notochord and in the apical ectodermal ridge of the developing limb of the mouse (Lyons et al, 1995). This suggests that heterodimers of these BMPs may play a part in these tissue interactions. In cultured rat marrow stromal cells (Aono et al, 1995) and insect cells (Hazama et al, 1995), increased induction of osteogenic properties in conditioned medium was seen when more than one BMP was expressed.

Signalling mediated by BMPs involves binding of the ligand to heterodimers of type I and type II receptors on the cell surface: a functional type I BMP receptor is necessary for effective induction of the mesoderm (Mishina et al, 1995). Under certain circumstances, other receptors can bind BMPs, for example in *Drosophila*, the punt gene product binds activin and not BMP-2. However, complexed with a type I receptor, BMP-2 is bound (Letsou et al, 1995).

In transfected COS cells, BMP-4 and BMP-7 were shown to bind to certain of the activin receptor-like kinase (ALK) family. It was shown that ALK-3 and -6 are type I receptors for BMP-4 and BMP-7 and also that ALK-2 is a type I receptor for BMP-7 but not BMP-4 (ten Dijke et al, 1994). The mRNA for these receptors have very different and restricted patterns of expression in the developing mouse: ALK-3 is found ubiquitously except for the liver, whereas ALK-6 is expressed later in development and in specific areas only, including the developing cartilage and bone. In the adult mouse, ALK-3 mRNA is found ubiquitously and that of ALK-6 is present only in the brain and



lung (Dewulf et al, 1995). These data indicate a wider role for BMPs in embryogenesis compared to that in the adult.

The signalling pathways mediated by BMPs are similar to those triggered by TGF-beta. In the intracellular signalling pathway initiated by the *Drosophila* dpp gene product, the functions of the maternally expressed genes Mad (Mothers against dpp) and Medea are indicated to have a temporal effect on the cascades of expression in the dpp pathways (Raftery et al, 1995). Receptor activation phosphorylates Mad-related proteins which are then translocated to the nucleus (Ferguson, 1996).

BMPs have been shown to use the mitogen-activated protein kinase (MAPK) signalling pathway: MAPK is phosphorylated by MAPK kinase (MAPKK) which was previously activated by MAPKK kinase (MAPKKK). It was demonstrated in mice that the activity of a protein kinase was stimulated upon treatment of the cell with TGF-beta or BMP, and was proposed to have a role in the regulation of transcription by TGF-beta. It was therefore suggested that this protein kinase may be a mediator in the signalling pathway of the TGF-beta superfamily (Yamaguchi et al, 1995).

### Calcitriol

The vitamin D endocrine system is pivotal to the regulation of bone and calcium homeostasis. Vitamin D<sub>3</sub>, a member of the steroid hormone superfamily, is formed by photoproduction in the skin and metabolised to its active form in the liver and kidney: it is hydroxylated at position 25 in the liver and then it is transported to the kidney where hydroxylation of position 1 $\alpha$  or 24R produces 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> or 24R,25-dihydroxyvitamin D<sub>3</sub> respectively (Tanaka et al, 1977). The most active naturally occurring metabolite of vitamin D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (calcitriol), has dramatic effects on the control of plasma calcium and phosphorus levels, and regulates the proliferation and differentiation of cells in a range of tissues including bone. It has been suggested that aging and sunscreen use coupled to inadequate dietary vitamin D can lead to osteomalacia and foster osteoporosis and that at least 400IU/day vitamin D plus at least 1000IU/day calcium are required to sustain good bone health (Holick, 1996). However, high doses of calcitriol can produce adverse physiological effects, including soft tissue calcification and hypercalcemia (Norman, 1995). Calcitriol is broken down upon further hydroxylation by 24-hydroxylase, the expression of which is upregulated by calcitriol itself (Nishimura et al, 1994) via interaction with two vitamin D response elements (VDREs) located within 263bp of the start site of transcription (Chen and DeLuca, 1995; Zierold et al, 1995).

### Genomic effects of calcitriol

The effects of vitamin D are mediated through a specific nuclear receptor, the vitamin D receptor (VDR), which is expressed in a wide variety of tissues including the pituitary gland, placenta, keratinocytes, colon, breast tissue and prostate gland, as well

as in the intestine, bone and kidney (Norman, 1995). The VDR binds to DNA in the form of a homodimer or a heterodimer with other members of the steroid hormone receptor subfamily, such as the retinoic acid, retinoid X or thyroid hormone receptors (RAR, RXR and THR respectively) (White et al, 1994). The mouse VDR gene was cloned and sequenced, revealing high homology with rat and human sequences in the DNA and ligand binding regions, with increased variability in the region between the two domains (Kamei et al, 1995): autosomal recessive calcitriol-resistant rickets can be caused by a mutation in the VDR (Hochberg and Weisman, 1995).

VDR expression was detected in the dental epithelium and mesenchyme of bud, cap and bell stages of the developing tooth germ, as well as in ameloblasts and odontoblasts (Bailleul-Forestier et al, 1996). Expression of COUP-TFs, members of the steroid hormone receptor superfamily and negative regulators of gene transcription (for example, of BMP-4), is regulated by retinoic acid (Qiu et al, 1996). They can bind to response elements of receptors of the steroid hormone superfamily, including receptors for vitamin D, thyroid hormone, retinoic acid and retinoid X. The transcription of a vitamin D-regulated gene, such as osteocalcin, may therefore depend on the ratio of COUP-TFs to positive regulators.

Calcitriol affects gene expression dose-dependently in chondrocytes: 10 nM calcitriol resulted in increased TGF-beta activity, and lower doses of calcitriol actively inhibited TGF-beta activity (Del Rio-Tsonis and Tsonis, 1994). Messenger RNA levels of TGF-betas 1 and 3 were found to be decreased, TGF-beta 2 transcription was raised (Farquharson et al, 1996) and N-cadherin expression was shown to decline with progression of chondrogenesis (Tsonis et al, 1994).

Pre-treatment of MG-63 osteoblastic cells with calcitriol transiently abolished the inhibitory effects of TGF-beta on alkaline phosphatase expression. This was shown to be due to the downregulatory effects of calcitriol on TGF-beta type II receptor levels (Iimura et al, 1994a). MG-63 cells were shown to express a variety of steroid hormone receptors, including those for glucocorticoid, retinoic acid and calcitriol. Vitamin D receptor (VDR) mRNA expression and stability, and protein expression was upregulated by calcitriol treatment; stability of VDR mRNA was unaffected by dexamethasone (dex) and increased by retinoic acid treatment (Mahonen and Mäenpää, 1994). Calcitriol treatment of the osteoblastic MG-63 cell line augmented expression of VDR and osteocalcin mRNA, and of secreted osteocalcin protein (Mahonen et al, 1990). Under mechanical strain, increased levels of bone matrix proteins such as osteopontin and osteocalcin are produced without the presence of an inducing hormone such as calcitriol. Treatment with calcitriol resulted in a further augmentation of the levels of these proteins (Harter et al, 1995). Calcitriol and TGF-beta act synergistically to increase type I collagen synthesis and alkaline phosphatase activity, but increased osteocalcin synthesis due to calcitriol treatment was ablated by TGF-beta treatment (Ingram et al, 1994).

As well as increasing expression of the VDR directly, calcitriol augments transcription and translation of its receptor indirectly using other signalling pathways. Phorbol 12-myristate 13-acetate (PMA) is a potent activator of the protein kinase C (PKC) pathway; treatment with PMA resulted in a time and dose-dependent increase in VDR expression in ROS 17/2.8 cells. PMA or calcitriol alone stimulated expression of VDR at the transcription and translation levels; co-treatment had a synergistic augmentation of VDR mRNA and protein expression which was not accompanied by increased affinity of VDR for calcitriol ligand. This evidence supports a link between VDR and PKC signalling pathway (Reinhardt and Horst, 1994).

#### Non-genomic effects of calcitriol

Calcitriol has been shown to have non-genomic effects in osteoblasts and chondrocytes, such as changes in arachidonic acid metabolism, prostaglandin production, protein kinase C activity and calcium ion flux. Cells which mineralise their matrix, such as chondrocytes, produce matrix vesicles which contain calcification enzymes, metalloproteinases and plasminogen activator. Vitamin D metabolites regulate the production of these vesicles, augment the expression of active and total metalloproteinase, and attenuate the expression of plasminogen activator and its activity by up to 75% in matrix vesicles isolated from growth zone chondrocyte cultures (Dean et al, 1996), facilitating their ability to activate latent TGF-beta (Boyan et al, 1994). It was postulated that activation of genomic and non-genomic pathways of calcitriol may be mediated by distinct receptors, as cells lacking the VDR still exhibit calcitriol-mediated effects and because the activation of the rapid non-genomic pathway is independent of the long-term effects on target genes (Khoury et al, 1995). Also, phospholipase C is activated and calcium channels are opened, suggesting that this is mediated by a receptor on the membrane (Baran et al, 1994).

Calcitriol induces cell-cell communication via gap junctions at low concentration; at higher levels of calcitriol, communication is suppressed, but this is reversed upon removal of calcitriol from the medium and replacement with trans-retinoic acid. Reciprocally, stimulation of intercellular communication by retinoic acid is blocked following removal of RA from the medium and consecutive treatment with calcitriol (Stahl et al, 1994).

Other bone formation stimulators include retinoids, fibroblast growth factors (FGFs), prostaglandins, platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), interleukins, thyroid hormone (T3) and parathyroid hormone (PTH) (Kang et al, 1995). Table 1.1 summarises the effects of several of these agents on gene expression in osteoblastic cells; table 1.2 illustrates the overall effects on osteoblastic proliferation and/ or differentiation.

### Specific aims of the project

MSX-2 expression is regulated by BMP-4 and BMP-2 and is associated with embryonic skeletal morphogenesis and development. There is some evidence to suggest that it is expressed in adult bone-derived cells. Therefore, a principal aim of this project was to investigate the role of MSX-2 in adult osteogenic differentiation, namely its expression and regulation in adult human bone-derived cells by osteogenic factors, including calcitriol and bone morphogenetic proteins (Chapter 4).

Osteoblastic cell culture systems are documented to provide models for the study of specialisation of osteoblasts, therefore a further aim was to investigate the appropriateness of using MG-63 osteosarcoma cells as a model of osteoblastic differentiation (Chapter 3).

Considered to be of great interest was to gain some understanding of the mechanisms by which calcitriol regulates MSX-2, and the other genes of interest, in comparison with the regulation of osteocalcin mRNA expression, which was used as a model of calcitriol-induced expression and as a marker of osteoblastic differentiation.

The importance of cell: cell and cell: matrix interactions with regard to osteoblast-related gene expression, and their modulation of the effects of osteogenic agents, were also evaluated.

Finally, to be examined were the effects of calcitriol and of the synthetic glucocorticoid dexamethasone, which is known to increase the incidence of osteoporosis with long-term use, on the differentiation of osteoblastic cells at different stages of maturation and on their pattern of gene expression (Chapter 5).

# The bone remodelling sequence (after Baron, 1990)

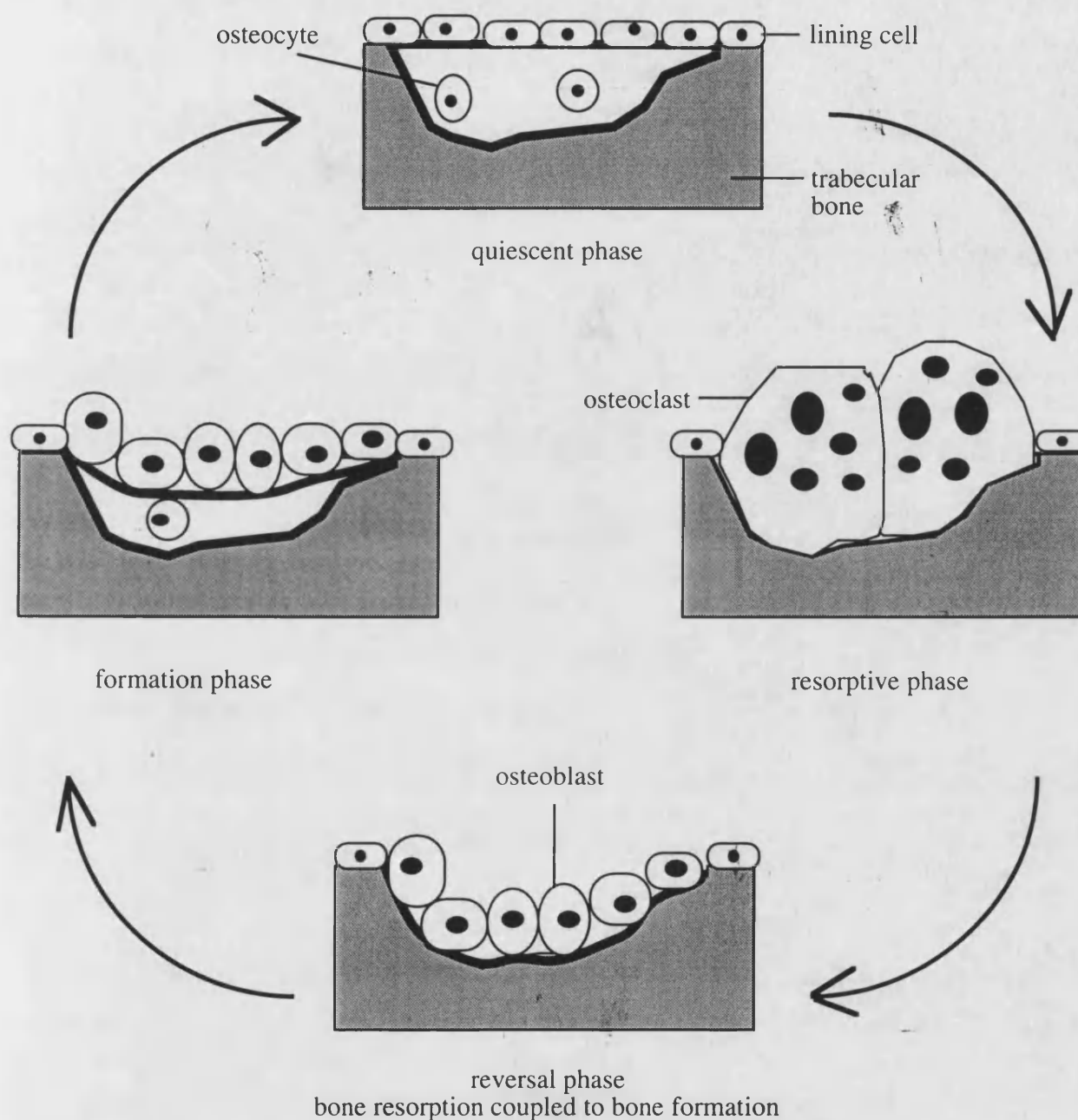


Figure 1.1

This diagram illustrates the bone remodelling sequence, where bone is initially in a resting phase, then osteoclasts are attracted to the site and resorption occurs. Osteoblasts replace the extracellular matrix, which then mineralises ten days later. Osteoblasts become either osteocytes or flat lining cells towards the end of the secretory period.

Diagrammatic representation of events associated with progression from bone marrow stem cell to fully differentiated phenotype  
(after Bruder et al, 1994)

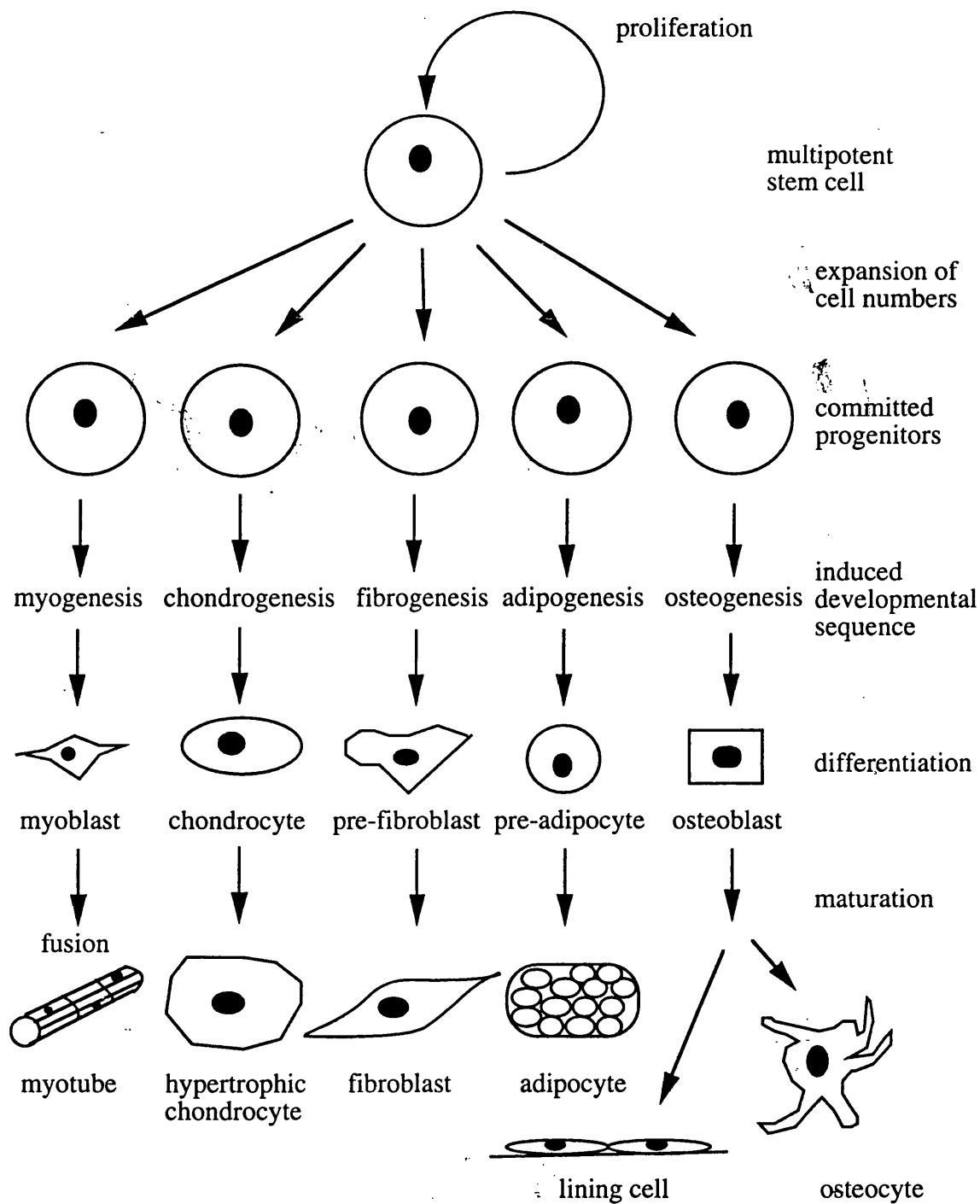
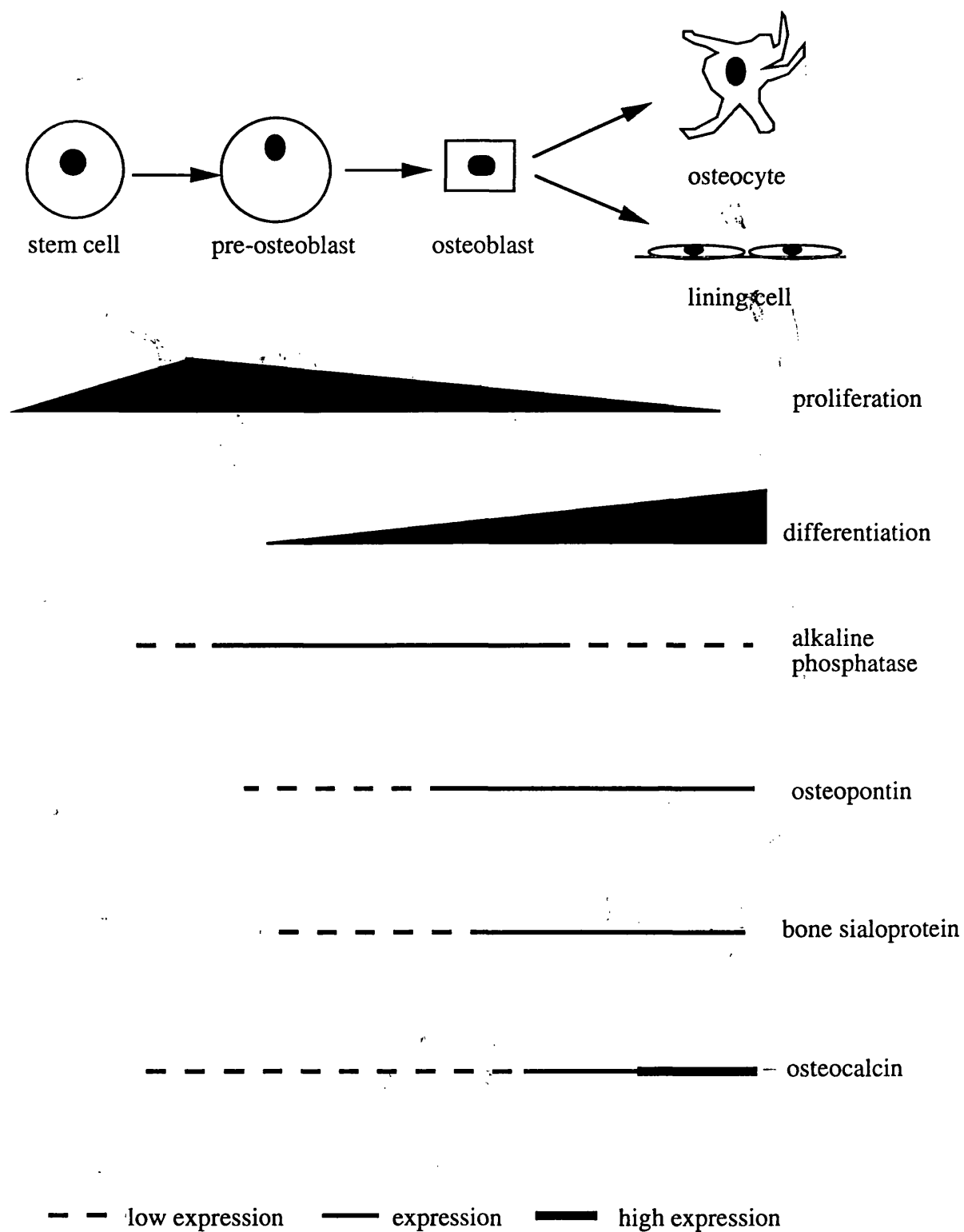


Figure 1.2

This figure is a simplified illustration of the putative progression of bone marrow stem cells to fully differentiated mature cells of the specified lineages. With advancing differentiation, the proliferative potential of the cell is reduced. However, evidence has been shown that diminished switching between phenotypes can occur under the appropriate conditions, for example, myoblast to osteoblast phenotype with BMP-2 treatment (Murray et al, 1993).

Figure 1.3 Sequential gene expression during osteoblast proliferation and differentiation (after Aubin et al, 1993)



Schematic representation of a long bone during development and of the growth plate  
(after Wallis, 1996)

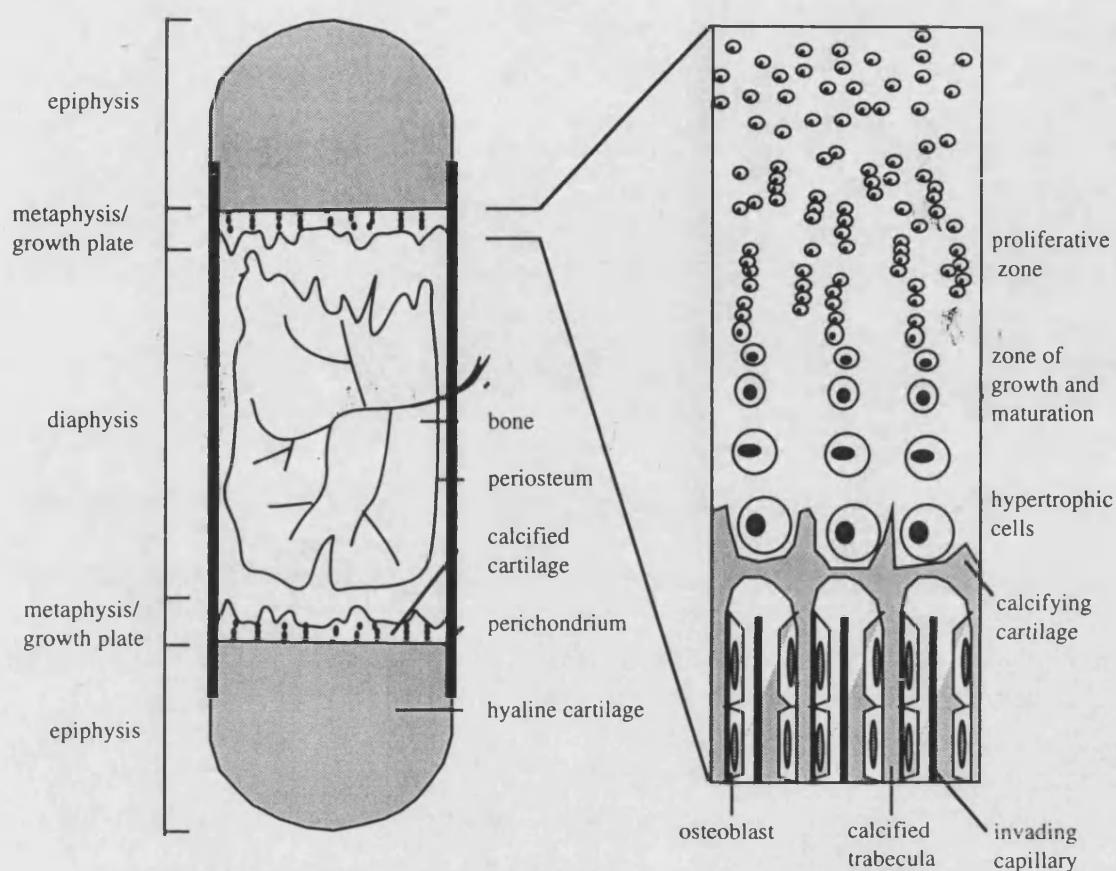
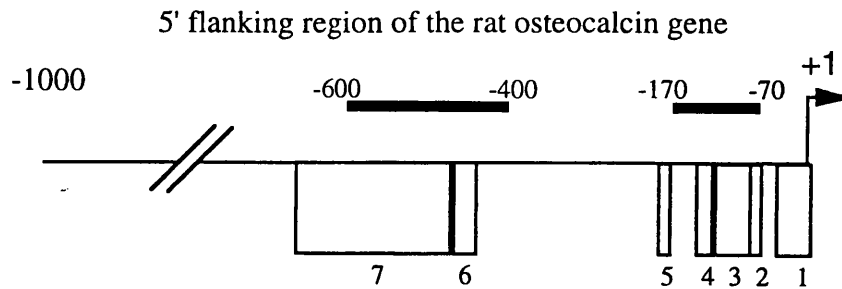


Figure 1.4

During embryonic development, chondrocytes in the centre of the cartilage template proliferate, then mature and hypertrophy. The surrounding matrix calcifies and osteoblasts invade, precipitating the replacement of cartilage with bone and bone marrow. This wave of replacement radiates outward with the resulting formation of the growth plates at the metaphyses, separating the cartilaginous epiphyses from the bony diaphysis. On the right is an illustration of a magnified growth plate, describing the progression of chondrocyte multiplication to hypertrophy and replacement by bone and marrow.



Figure 1.5 The regulation of osteocalcin transcription



1 -33 GATATAAAAGGTATTGCAGAACAGACAAGTCCC -1

The sequence contains a TATA box (underlined) and a glucocorticoid response element (boxed, with the hexameric half-sites underlined)

- 2 Included within the 28bp sequence located at -74 to -47 is the OSE1 element (sequence below), which is activated also in osteoclastic cells early in their differentiation

5' CCTCCTGCTTACAT 3'

- 3 This region is a primary transcription regulatory sequence. From -121 to -74, it has been designated a cAMP response region. It encapsulates the osteocalcin box (OC box, -102 to -76, boxed), within which are several consensus binding sites: AP-1 (small boxes), Msx (underlined), CAAT box (heavy overline), E box (overline) and steroid hormone half-sites (heavy underline).

Also binding here was an osteoblast-specific binding protein (OCBP) whose binding sequence overlaps with that of MSX.

-121 TTGGGTTTGACCTATTGCC CACATGACCCCAATTAGTCCT GGCAGCA -74

- 4 The OSE2 sequence (-146 to -132) binds an osteoblast-specific factor which activates osteocalcin gene expression only in mature, mineralising osteoblasts. Contained within this region is the consensus binding sequence for members the runt family of core-binding factors. CBFA1, a runt domain-containing transcriptional activator, is essential for the normal formation of bone and binds the osteocalcin promoter at the following sequence within the OSE2:

5' CCAACCACA 3'

- 5 Basal transcription is regulated in part at least through a CP-1 binding motif located between -200 and -180.

5' TCTGATTGTGT 3'

- 6 A vitamin D response element (boxed with the hexameric half-sites underlined), two binding sites for nuclear matrix proteins (overlined) and an AP-1 site (heavy underline) are located between -465 and -434:

5' GCACT GGGTGAATGAGGACA TTACTGACCGCT 3'

- 7 Element(s) associated with upregulation of osteocalcin expression following FGF-2 treatment are thought to bind in the region between -678 and -476

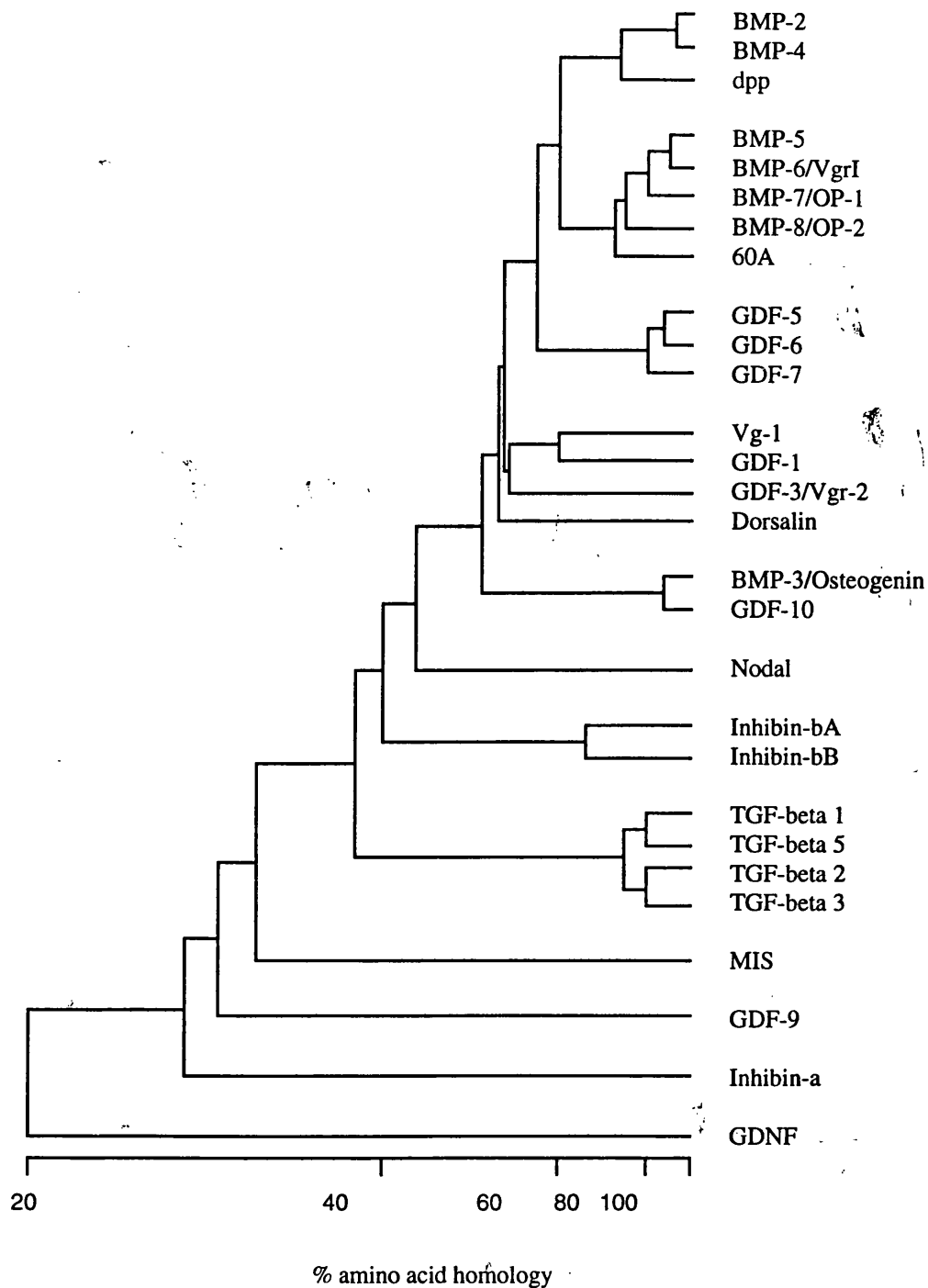


Figure 1.6

The transforming growth factor-beta superfamily contains several subfamilies which are grouped according to their amino acid sequence homology. Included are members whose sequences and functions are highly conserved in different species for example Bmp-2 and Bmp-4 (human) and dpp (Drosophila).

Table 1.1 Effects of hormones and growth factors on osteoblastic gene expression

<u>Gene</u>	<u>Agents upregulating gene expression</u>	<u>Agents downregulating gene expression</u>
osteocalcin	BMPs <sup>4, 37</sup> calcitriol <sup>1, 19, 24, 32, 39, 44, 45</sup> ascorbic acid <sup>31</sup> dex <sup>44, 45</sup>	TGF-beta <sup>23, 43</sup> dexamethasone (dex) <sup>28, 29</sup>
osteopontin	TGF-beta <sup>13, 20, 23</sup> calcitriol <sup>13, 20, 32, 39, 44, 45</sup> retinoic acid <sup>20</sup> FGF-2 <sup>20</sup> dex <sup>44, 45</sup>	TGF-beta <sup>20, 23</sup>
bone sialoprotein	dex <sup>20</sup>	calcitriol <sup>20</sup>
type I collagen	FGF-2 <sup>25</sup> TGF-beta <sup>2, 18, 23</sup> ascorbic acid <sup>31</sup>	calcitriol <sup>15, 45</sup> TGF-beta <sup>23</sup> dex <sup>28</sup>
VDR	calcitriol <sup>12, 39, 40</sup> retinoic acid <sup>9, 10, 40</sup> dex <sup>11, 40</sup>	
TGF-beta	calcitriol <sup>8, 30, 36</sup> retinoic acid <sup>42</sup> TGF-beta <sup>36</sup>	retinoic acid <sup>36</sup>
MSX genes	BMPs <sup>4, 5, 21, 34</sup> calcitriol <sup>35</sup>	dex <sup>46</sup>
BMPs	BMPs <sup>26</sup> calcitriol <sup>37</sup>	
alkaline phosphatase	BMPs <sup>4, 7, 33, 37</sup> TGF-beta <sup>42</sup> ascorbic acid <sup>31</sup> dex <sup>45</sup>	calcitriol <sup>45</sup> TGF-beta <sup>23, 37, 42</sup>

Where agents appear in both columns, their effects are biphasic, depending on the maturation stage of the cells when treated and/or upon the dose of the agent used.

Table 1.2 Agents affecting proliferation/differentiation of osteoblasts

<u>Increased proliferation</u>	<u>Increased differentiation</u>
TGF-beta	TGF-beta <sup>23, 24, 28, 43</sup>
FGF-2	FGF-2 <sup>17</sup>
thyroid hormone	thyroid hormone <sup>15</sup>
parathyroid hormone	parathyroid hormone <sup>6, 18</sup>
prostaglandins	prostaglandins <sup>3</sup>
	BMPs <sup>4, 7, 34, 38</sup>
	calcitriol <sup>45</sup>
	retinoic acid <sup>43</sup>
	dexamethasone <sup>45</sup>

As before, where agents appear in both columns, their effects are biphasic, depending on the maturation stage of the cells when treated and/or upon the dose of the agent used.

<sup>1</sup> Araki et al, 1993; <sup>2</sup> Centrella et al, 1991; <sup>3</sup> Raisz and Fall, 1990; <sup>4</sup> reviewed by Hogan, 1994; <sup>5</sup> Graham et al, 1994; <sup>6</sup> Hruska et al, 1993; <sup>7</sup> Iwasaki et al, 1994; <sup>8</sup> Koli and Keski-Oja, 1995; <sup>9</sup> Petkovich et al, 1984; <sup>10</sup> Chen and Feldman, 1985; <sup>11</sup> Chen et al, 1986; <sup>12</sup> Costa et al, 1985; <sup>13</sup> Noda et al, 1988; <sup>14</sup> Ohishi et al, 1994; <sup>15</sup> Pavlin et al, 1994; <sup>16</sup> Pitaru et al, 1993; <sup>17</sup> Nishida et al, 1994; <sup>18</sup> Thiébaud et al, 1994; <sup>19</sup> Ozono et al, 1991; <sup>20</sup> reviewed by Young et al, 1993; <sup>21</sup> Barlow and Francis-West, 1997; <sup>22</sup> reviewed by Bonewald and Mundy, 1989; <sup>23</sup> Breen et al, 1994 a; <sup>24</sup> Breen et al, 1994 b; <sup>25</sup> Canalis et al, 1988; <sup>26</sup> Ghosh-Choudhury et al, 1996; <sup>27</sup> Centrella et al, 1989; <sup>28</sup> Delany et al, 1994; <sup>29</sup> Beresford et al, 1984; <sup>30</sup> Farquharson et al, 1996; <sup>31</sup> Franceschi and Iyer, 1992; <sup>32</sup> Harter et al, 1995; <sup>33</sup> Hughes et al, 1995; <sup>34</sup> Iimura et al, 1994; <sup>35</sup> Hodgkinson et al, 1993; <sup>36</sup> reviewed by Kim and Ballock, 1993; <sup>37</sup> reviewed by Wozney, 1993; <sup>38</sup> Lian and Stein, 1992; <sup>39</sup> Mahonen et al, 1990; <sup>40</sup> Mahonen and Mäenpää, 1994; <sup>41</sup> reviewed by Massagué, 1996; <sup>42</sup> reviewed by Moses and Serra, 1996; <sup>43</sup> Piskanen et al, 1994; <sup>44</sup> Pockwinse et al, 1995; <sup>45</sup> reviewed by Stein and Lian, 1993; <sup>46</sup> Towler et al, 1994.

## **Chapter 2**

### **MATERIALS AND METHODS**

## MATERIALS

### 1. Human bone explants

Bone samples were collected from patients undergoing surgery for fracture or joint replacement from the Bristol Royal Infirmary, Frenchays Hospital and from the University of Bristol Medical School. These were stored in sterile sealed bags or in flasks containing sterile medium at 4°C until use.

### 2. Human osteosarcoma cells (MG-63)

These were stored in liquid nitrogen in aliquots of 1 ml, sufficient to seed 2 T<sub>75</sub> flasks. They were obtained from the European Collection of Animal Cell Cultures (ECACC) at passage 107.

### 3. Rat osteosarcoma cells (ROS 17/ 2.8) and mouse calvaria-derived cells (MC3T3-E1)

These were stored in liquid nitrogen in aliquots of 1 ml and were supplied by the American Tissue Culture Collection (ATCC).

### 4. Tissue culture reagents

These were generally supplied by Gibco BRL, unless otherwise stated.

### 5. FACS antibodies

Hybridomas for the monoclonal mouse anti-human antibodies Stro-1 and AP (B4-78) were supplied by the Developmental Studies Hybridoma Bank, University of Iowa (Stro-1, Simmons and Torok-Strob, 1991; AP, Lanson et al, 1985). These were cultured and the supernatants, containing secreted antibody, were collected for use in the FACS analysis: the alkaline phosphatase antibody was purified by protein G column separation by Dr. Carolyn Jefferiss. IgG<sub>1</sub> was supplied by Sigma and OB/L was raised in-house by Dr. Susan Walsh. The goat anti-mouse human adsorbed secondary conjugates anti-IgG<sub>1</sub> R-PE and IgM FITC were supplied by Southern Biotechnology Associates Inc. in 1ml quantities.

### 6. Bacterial strains

The E. coli strain DH5 $\alpha$  was used for plasmid amplification and was supplied by Promega.

### 7. Bacterial growth media

Bacterial growth medium components were supplied by Unipath Ltd. and Sigma.

#### 8. Cloning vectors

The phagemid pBluescript was used as a vector for a 2.6 kb MSX-2 insert and was supplied as containing the insert by Professor Paul Sharpe, UMDS, London.

251 bp of the propeptide coding region of BMP-2 were cloned into the pSP65 vector and 500 bp of the propeptide coding region of BMP-4 were cloned into pSP64, and supplied as containing the insert by John Wozney, Genetics Institute, Cambridge, Massachusetts.

The vector pAT153 containing the 2.6 kb insert for alkaline phosphatase was supplied to us by Mitchell Weiss at the University of Pennsylvania (Weiss et al, 1986).

The sequence for a 40 base oligonucleotide which binds to the coding region of osteocalcin was selected by J. Beresford and made originally at the MRC Molecular Haematology Unit, John Radcliffe Hospital, Oxford by J. Lamb. The sequence reads: 5' - CCA ACT CGT CAC AGT CCG GAT TGA GCT CAC ACA CCT CCCT -3'

A 1 kb fragment spanning the entire amino acid coding region of GAPDH was cloned into pUC18 (Beresford et al, 1992).

#### 9. Enzymes

Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs Ltd., Promega Ltd., Gibco BRL, Northumbria Biologicals Ltd., Boehringer Mannheim or Pharmacia Biotech and were used in accordance with manufacturer's instructions.

#### 10. Radioisotopes

<sup>32</sup>P-labelled radioactive isotopes (dATP and dCTP) were supplied in aqueous 10 µCi/ µl solution by Amersham Life Sciences UK Ltd.

#### 11. General laboratory chemicals

All chemical reagents used in the making of buffers and other compounds were obtained from BDH Ltd. or Sigma Ltd. unless otherwise indicated and were of molecular biology or AnalaR grade.

## **METHODS**

### **2.1 TISSUE CULTURE**

#### **2.1.1 Isolation and culture of cells from explants of human trabecular bone**

Bone fragments were handled in a Class 2 hood only and were cut into smaller fragments using sterile instruments. Fragments were kept moist at all times by immersing the bone in sterile phosphate buffered saline (PBS). Forceps were used to hold the bone explant in place while small pieces of trabecular bone were cut from it using a scalpel or scissors. These pieces were then cut down again into fragments of 2x 2x 2 mm or less. These were placed in a 50 ml Falcon tube with 20 ml fresh PBS and vortexed vigorously for 15 seconds. The bone chips were allowed to settle and the PBS was removed carefully (with a pipette if a large amount of fat was present). The chips were washed in PBS a further 4-5 times or until all contaminating blood cells and marrow tissue was removed. The chips were transferred to 75 cm<sup>2</sup> tissue culture flasks and 15 ml Dulbecco's modified Eagle's medium (DMEM) (see Appendix 1) plus 10% fetal calf serum (heat-inactivated FCS, Globepharm) was added. The cultures were maintained in an incubator at 37°C in a humidified atmosphere of 95% air: 5% CO<sub>2</sub> and were fed at days 7 and 14. After this, the cultures were fed every 3-4 days until near confluence (usually 5-6 weeks) when cultures were fed every 2-3 days.

#### **2.1.2 Isolation and culture of cells derived from human marrow**

Samples of bone were handled in sterile conditions as for trabecular explants. All connective tissue and periosteum was removed with a scalpel blade to prevent contamination of the culture with fibroblasts and other cells. The marrow was suspended in serum-free DMEM and spun at 400g for 5 minutes in a benchtop centrifuge (Beckman centrifuge, model GPR) before the supernatant was removed. The cell pellet was resuspended in 25 ml of DMEM which were then layered onto 20 ml Lymphoprep in a 50 ml Falcon tube. This was then centrifuged at 500g for 30 minutes with the brake off, so as not to disturb the final cell layers. The mononuclear cells were collected by removing the interface to a fresh tube whereupon 10 ml of DMEM were added and the cells spun again at 250g for 10 minutes to wash out the Lymphoprep. Cells were resuspended in 15 ml DMEM and passed through a 70 µm cell filter (Falcon) to remove clumps. The filter was washed through with DMEM and the wash added to the cell suspension.

Viability was examined prior to plating by taking a small sample of cell suspension and adding Trypan blue:

60 µl DMEM

20 µl cell suspension

20 µl Trypan blue

(0.5% solution in 0.85% saline, Flow Laboratories Inc.)



The mix was allowed to stand for 2 minutes, then a small amount was loaded into a haemocytometer and counted. Any cells staining blue were counted as non-viable and cells remaining white were counted viable. The method for counting was thus: cells lying within the boundaries of the grid were counted. Any that were lying on the boundaries to the left vertically or to the top horizontally were also included. Those cells lying on the vertical right or bottom horizontal were not included. A count was taken until at least 80 viable cells had been counted, then this number was multiplied by  $10^4$  to account for the dimensions of the haemocytometer and again by 5 as this was the dilution factor.

Cells were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> and left to establish in an incubator for 3 days. At this point, non-adherent cells were removed by washing twice in PBS and the medium replaced. Cells were fed with fresh medium every 3-4 days thereafter for 2 weeks at which point the cells were redistributed following collagenase and trypsin treatment. Incubation was continued until confluency was reached.

#### 2.1.3 Culture of human, mouse and rat osteoblast-like cell lines

MG-63, MC3T3-E1 and ROS 17/2.8 cells were removed from storage in liquid nitrogen and heated rapidly to 37°C by placing them in a waterbath or incubator. An aliquot of 1 ml was split between 2 75 cm<sup>2</sup> flasks and 15 ml DMEM plus 10% FCS were added (MC3T3-E1 cells were cultured constantly in the presence of 50 µM ascorbate-2-phosphate). The contents of the flasks were swirled gently to mix and the cells were incubated overnight as above. The following day, the medium was removed and fresh culture medium was added so that the high concentration of dimethyl sulphoxide (DMSO) from the storage medium would not affect the cells. Thereafter, the cultures were fed every 2-3 days until confluency (10-14 days).

#### 2.1.4 Passaging of cultured cells

Culture medium was removed from the flask and the cell layer was washed by addition of PBS to an opposite corner and gentle swirling. The supernatant was then removed and the cell layer washed twice more.

Trabecular bone explant-derived cell cultures only:

25 units collagenase IV/ml DMEM supplemented with 2 mM CaCl<sub>2</sub> were added following removal of the final PBS wash and the flask was incubated in culture conditions for approximately 40 minutes. At this stage, the cell layer was examined by phase-contrast microscopy (Wilovert) to ensure that necrosis had not occurred, that the cell layer was not peeling from the flask surface and that the extracellular matrix was being digested. The cell layer was again washed three times in PBS.

All cell cultures:

5 ml trypsin-EDTA (Gibco BRL) was added per 75 cm<sup>2</sup> flask following removal of the final PBS wash. The flask was incubated under culture conditions for 5-10 minutes and examined by phase-contrast microscopy to ensure that the cell layer was being removed from the flask surface. A cell suspension was produced by gently pipetting up and down repeatedly with a sterile 2 ml pipette to break down large clumps of cells. The suspension was transferred to a fresh 50 ml Falcon tube containing 200-500 µl fetal calf serum (FCS, Globepharm) to stop the action of the trypsin.

The cell suspension was centrifuged at 200g (Beckman centrifuge, model GPR) for 10 minutes at 4°C with the brake off so that the pellet would not be disturbed by rapid halting. The supernatant was removed and 2 ml fresh culture medium containing 20 units/ml DNase I were added to the pellet. Resuspension of the pellet was achieved by gently pipetting up and down with a sterile 2 ml pipette. The suspension was removed to a fresh Falcon tube with a 70 µM filter (Becton Dickinson) attached, removing fine bone chips. The filter was washed through with 2 ml DMEM plus DNase I and the suspension was made up to 2 ml DMEM for every flask plus 2 ml.

Cells were counted using a haemocytometer (see section 2.1.2) and seeded in the range  $5 \times 10^3$  to  $5 \times 10^4$  cells per cm<sup>2</sup> flask. Medium was replaced every 3-4 days until confluency at which stage the cells were treated.

#### 2.1.5 Storage of cell lines in liquid nitrogen

Following passaging of cultures, a small quantity of cells were stored to maintain the stocks. Sufficient cells to seed 2 T<sub>75</sub> flasks were made up in 10% DMSO and 50% FCS and the tubes were then placed at -20°C for a few hours, then at -70°C overnight, then finally into liquid nitrogen.

#### 2.1.6 Treatment of cells with exogenous growth factors and morphogenetic proteins

Cells were fed at least 24 hours prior to treatment in an attempt to abolish the effects of early response genes. The serum content of this replacement medium was dropped from 10% to 2%. Cells were treated with BMP-2, BMP-4, BMP-6, BMP-7, TGF-beta 1, FGF-2 and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (calcitriol) and the following investigations were performed:

- a) dose response, in which a range of doses of these factors has been applied to cultures to discover the most effective dose for induction/repression of specific gene expression;
- b) time course, in which the effective dose of these factors has been applied to cultures over varying time intervals to determine when the most dramatic effects of these factors are; and,

c) interaction studies, where various combinations of these factors have been introduced to cultures to examine the effects of two or more factors simultaneously on the expression of specific genes.

The ranges used for each factor were:  $10^{-7}$ - $10^{-11}$  M for calcitriol; 5- 200 ng/ ml final concentration for BMP-2 and BMP-7; 5- 150 ng/ ml final concentration for BMP-4 and BMP-6; and, 0.05- 1.25 ng/ ml TGF-beta. FGF-2 was used at 2.5 ng/ ml final concentration as this was found previously to produce an effect. The time intervals used were from 30 minutes to 24 hours generally but in some cases longer exposures to the factors were appropriate.

For the BMPs, FGF-2 and TGF-beta, a small sample of the stocks was serially diluted in DMEM plus FCS, the appropriate quantity of diluted factor being added directly to medium in the flask and mixed by gentle swirling. For calcitriol, the same procedure was carried out using a Hamilton syringe in minimal lighting conditions as calcitriol is sensitive to light. The flasks were incubated for their accorded time and then prepared for extraction of their nucleic acids.

## 2.2 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

### 2.2.1 Principle and applications of flow cytometry

Flow cytometry is a sophisticated technique which enables the swift measurement of single particles as they pass individually in a fast-flowing stream through a detector. Flow cytometers have the capabilities to measure a wide range of cellular parameters based on light scatter and fluorescence and can separate cells into subpopulations depending on the parameters selected. This latter application is known as cell sorting (Carter and Meyer, 1990). The properties chosen for measurement can include enzyme activity, pH and nucleic acid content, and with the use of monoclonal or polyclonal antibodies conjugated to fluorescent dyes, cell surface markers such as receptors can be tagged and their density and distribution gauged. Cell sorting involves the use of electrostatic deflection of droplets, whereby the stream of cells is vibrated and a voltage applied resulting in droplets, each containing a single cell, breaking off in a stream with a positive or negative electric charge. The voltage is applied as a pulse, which coincides with the formation of the droplet containing the cell. The delay between the initial detection of the cell and its containment within the droplet is set exactly and therefore only the desired cells will be charged and collected: they are separated from the stream when they pass through the electrostatic field formed between high voltage plates (see figure 2.1). Two subpopulations can be collected simultaneously with the rest of the cells going to waste collection.

Fluorescence is a result of electrons returning to a ground state by the emission of a quantum of light following excitation of the original molecule. The wavelength of the emitted light will be longer than that of the absorbed light as it will be of lower

energy. Therefore, they can be separated from each other with the use of optical filters which results in the detection of a positive signal against a negative background.

Two important properties of fluorescent compounds are the extinction coefficient, the quantity of light absorbed at a given wavelength, and the quantum efficiency, the number of photons emitted for every photon absorbed. The absorption maximum should be at a wavelength close to one of the spectral lines of the laser used in the flow cytometer; the argon-ion laser used in these experiments produced light at 488 nm wavelength. If the quantum efficiency is high, a strong fluorescent signal will be obtained (Ormerod, 1990). Fluorescein isothiocyanate (FITC) and R-phycoerythrin (R-PE) are two of the most widely used fluorescent labels (fluorophores/fluorochromes) today because they can both be excited at 488 nm. When two fluorophores are used in a dual labelling system, overlap between the emission spectra must be avoided to increase the efficiency of the sort - the emission maxima of FITC and R-PE are 520 nm (green light) and 576 nm (orange light) respectively. Therefore, there may be some level of crosstalk between the two dyes which must be compensated for in the FACS process.

### 2.2.2 Labelling of samples

It is thought that trabecular and marrow-derived bone cells express Stro-1 and alkaline phosphatase (AP) proteins on their cell surfaces at progressive stages of maturation (Stewart et al, 1996). In order to determine at which stage(s) of maturation the genes of interest in this investigation were expressed, cells were sorted according to the presence or absence of either or both Stro-1 or AP.

Cells were cultured to confluency and passaged as described in section 2.1.3 until after the stage of counting by haemocytometer. At this point, the cells from each treatment group were aliquoted into FACS tubes (Falcon) as follows:  $10^5$  -  $2 \times 10^5$  cells into each of three tubes designated negative control (no antibody labelling), Stro-1 (control Stro-1 antibody labelling only) and AP (control AP antibody labelling only) - the cells in these tubes were necessary to set up the parameters for sorting. The rest of the sample was placed in a separate tube for dual labelling. The tubes were centrifuged at 200g for 10 minutes with the brake off (Beckman centrifuge, model GPR) and the supernatant was removed carefully. Cells were resuspended in blocking buffer (see Appendix 1), 500  $\mu$ l for controls and 5 ml for dual labelling, and incubated in ice for 15 minutes. Cells were pelleted by centrifugation and the supernatant removed. The cell pellets were resuspended in primary antibody as follows - quantities for the controls are for  $10^5$  cells, and for dual labelling samples, quantities are for  $10^7$  cells:

sample \ antibody	OB/L	Stro-1	IgG <sub>1</sub>	AP
negative control	100 $\mu$ l	-----	10 $\mu$ g/ ml	----
Stro-1	-----	100 $\mu$ l	10 $\mu$ g/ ml	----
AP	100 $\mu$ l	-----	-----	10 $\mu$ g/ ml
dual labelling	-----	2 ml	-----	10 $\mu$ g/ ml

The suspended cells were incubated in ice for 45 minutes, then HHF (HBSS, 10mM Hepes, 5% FCS - see Appendix 1) was added up to 4 ml. The cells were centrifuged for 10 minutes at 200g with the brake off and the supernatant was removed. Cells were then resuspended in secondary antibody (conjugates): 1/200 anti-IgG<sub>1</sub> R-PE and 1/25 anti-IgM FITC were made up in HHF and 50 µl was added to the controls and 1 ml to the dual labelling cell pellets. The tubes were again incubated in ice for 45 minutes before addition of HHF to 4 ml and subsequent centrifugation. Control cells were resuspended in 500 µl HHF and the dual labelling cells were resuspended in 2 ml HHF. They were then stored in ice for immediate FACS analysis.

### 2.2.3 Cell sorting

The flow cytometer (FACStar plus, Becton Dickinson) was switched on a couple of hours before use to allow the machine to warm up adequately. The system was flushed through to ensure the removal of clumps of cells, debris or air bubbles, and the droplet delay was set. The cell sample was filtered again just before analysis to ensure removal of clumps and so avoid blocking the machine. The samples were put through the flow cytometer in the same order as labelled (see section 2.2.2). The Stro-1 sample fluorescence (FL-2) was shown in the vertical plane on the monitor. The fluorescence for the AP sample (FL-1) was shown in the horizontal plane. Compensation for the cross-talk fluorescence was adjusted where necessary to ensure efficient separation of the four subpopulations of cells.

The dual labelled sample was taken into the flow cytometer. A count of  $10^4$  cells was taken first as an idea of the distribution of cells between the subpopulations, then it was decided which populations to collect first. Sample tubes were labelled with the subpopulation to be collected: -/- for cells not labelled with Stro-1 or AP; +/- for cells labelled only with Stro-1; ++ for those labelled with both antibodies; and -/+ for AP positives only. 500µl HHF were added to each collection tube, two of which were placed in the collection holders. Up to  $3 \times 10^5$  cells for each subpopulation, where possible, were obtained and the tubes were placed in ice until the FACS was completed. For those subpopulations for which sufficient cells were collected, a sample was reanalysed to assess purity of the sort. All data were stored onto a disk formatted in BD HP\*2 to facilitate conversion and analysis.

### 2.2.4 Treatment of cells

The cell suspension was removed into a fresh 1.5 ml tube and the cells were pelleted at 2000 rpm for 5 minutes in a benchtop centrifuge. The supernatant was removed carefully and the sealed tube placed briefly in liquid nitrogen to snap freeze the cell pellet. The tube was then stored at -70°C unless RNA was to be extracted immediately from the cells.

### 2.2.5 Analysis of data

The disk was labelled with the appropriate information and taken to a Quadra 650 system (Macintosh) for conversion. The application Consort File Exchange (Becton Dickinson) allowed translation of the data to a format readable by the machine. They were converted by use of the FACSCConvert file where the data became readable by the CellQuest program, within which final analysis of the FACS data occurred.

At this point, the data were plotted as a dot plot of FL-1 readings (AP) against FL-2 readings (Stro-1). The sample was gated using the negative plot, so that less than 5% of cells were present in any of the positively labelled quadrants. This gate was pasted onto the singly and dual labelled plots to ensure consistency and so that background could be removed. This also facilitated comparisons with other treatment groups in the same experiment. Sectors representing the four collected subpopulations were added onto the dual labelled sample. These four rectangles were copied and automatically placed into the corresponding areas on the other plots from the same treatment group so that distributions of cells between the four subpopulations could be compared in the dual labelled sample. Statistical analysis of the data was also performed in the same program, supplying percentages of the total number of cells present in each subpopulation region.

## 2.3 PREPARATION OF NUCLEIC ACIDS FROM BONE AND OSTEOSARCOMA CELL CULTURES

### 2.3.1 Extraction of RNA from cultured cells

RNAzol™ B (Biogenesis Ltd.) solution contains both guanidinium thiocyanate and phenol and can be used as a simple one-step extraction for RNA: cells can be lysed directly in RNAzol™ B, promoting the formation of RNA complexes with guanidinium thiocyanate and water, while simultaneously abolishing the hydrophilic interactions of DNA and proteins. Thus, DNA and proteins are effectively removed to the organic phase leaving only RNA present in the aqueous phase (after Chomczynski and Sacchi, 1987).

Cells were passaged as detailed above until just following the centrifugation stage. Once the cells were spun down, the pellets were washed twice with PBS. The sample were lysed in 200 µl RNAzol™ B and sealed, then vortexed briefly before addition of 20 µl chloroform. The samples were again sealed tightly and vortexed for 15 seconds then chilled at 4°C for 10 minutes. The suspensions were centrifuged at 12000g for 15 minutes at 4°C (model GS-15R refrigerated centrifuge, Beckman).

At this point, the homogenate formed two layers: an upper colourless aqueous layer containing the RNA, and the lower blue organic phase. A white interphase was also usually present indicating an excessive amount of protein present in the homogenate. The aqueous phase was transferred to a fresh tube and an equal volume of

chilled isopropanol was added. The samples were then stored at 4°C for 15 minutes prior to a second centrifugation at 12000g for 15 minutes at 4°C.

The supernatant was removed carefully with a pipette and the samples inverted and allowed to air dry for 10 minutes before addition of 50 µl water. The RNA pellets were allowed to resuspend at 4°C for 16 hours. If resuspension was unsuccessful, the RNA was heated to 60°C for 10 minutes before storage at -70°C, unless the RNA was to be used immediately in a reverse transcription reaction.

This approach was found to have limited applications as, while suitable for reverse transcription (see 2.3.2) and polymerase chain reaction amplification (see 2.4), the RNA was generally not of sufficient purity for Northern blot analysis (see 2.7.2). This may have been due to co-purification of proteoglycans. To obtain extremely clean RNA, it was necessary to use a more extensive method.

Human trabecular bone-derived cells were passaged as above, and at the pellet stage following trypsinisation, the cells were washed twice with PBS and re-pelleted. In the case of cultured cell lines, medium was removed and the monolayer was washed three times in PBS. For cells in a T<sub>75</sub> flask, 8 ml of the following lysis solution were added; for cell pellets derived from FACS analysis, 500 µl were used per tube:

4.22 M	guanidine thiocyanate
0.026 M	sodium citrate
0.53%	sodium lauroyl sarcosine

To 20 ml of lysis solution were added 144 µl 2-mercaptoethanol just prior to use. Cells were suspended in this lysis solution by pipetting and the suspension was transferred to a 50 ml Falcon tube to which were added:

0.09 M	sodium acetate, pH4.2
8 ml	water-saturated phenol
1.6 ml	chloroform

The contents of the tube were vortexed for 30 seconds and placed on ice for 15 minutes. The mixture was transferred to a baked glass Corex tube and centrifuged for 15 minutes at 10000 rpm in a SA600/SS34 rotor (Beckman). The aqueous supernatant was removed to a fresh 50 ml Falcon tube to which 7 ml ice-cold isopropanol were added. The RNA was allowed to precipitate overnight at -20°C.

The suspension was centrifuged for 15 minutes at 5000 rpm, then the supernatant was removed. The pellet was dissolved in 2 ml DEPC-treated water (Appendix 1) and 2 ml phenol/ chloroform (1:1) were added. The mixture was vortexed for 10 seconds then centrifuged for 5 minutes at 3000 rpm. The supernatant was removed to a fresh 15 ml Falcon tube. 0.68 M ammonium acetate and 90% ethanol

were added and the RNA was allowed to precipitate overnight at -20°C. The following day the tube was centrifuged for 5 minutes at 5000 rpm, the supernatant was removed and the pellet was washed in 70% ethanol. All liquid was removed and the pellet was allowed to air dry for 10 minutes before dissolution in an appropriate volume of DEPC-treated water. RNA was quantified by UV spectrophotometry (Ultrospec II, LKB Biochrom) and stored at -70°C until use in Northern blot analysis (see 2.7.2).

### 2.3.2 Preparation of cDNA from RNA

The effects of factors on gene expression can be examined in several ways following extraction of the RNA. In this investigation, two methods have been introduced: reverse-transcription polymerase chain reaction (RT-PCR) and Northern analysis (see section 2.7.2).

Before low levels of RNA can be amplified, they must first be reverse transcribed to cDNA so that the DNA-dependent DNA polymerase used in the PCR will have a template. cDNA synthesis was carried out as follows:

5 µl	5x first strand buffer (Gibco BRL)
0.2 U	random hexamer primers [Pd(N) <sub>6</sub> ] (Pharmacia Biotech)
8 mM	DTT (Gibco BRL)
0.8 mM	dNTP mix (supplied as 100 mM each nucleotide, Pharmacia Biotech)
100 U	reverse transcriptase (Superscript RT, 200 U/ µl, Gibco BRL)
20 U	RNasin (RNase inhibitor, 40 U/ µl, Promega)

This was made up to 20 µl with distilled water and 25 µl mineral oil was overlaid. 5 µl RNA was added through the oil layer and mixed by gently flicking the tube. Samples were checked for genomic contamination by setting up parallel reactions in the absence of reverse transcriptase.

Optional at this point was removal of 5 µl to a fresh tube and the addition of 0.5 µl radiolabel to this to monitor the incorporation efficiency of the reverse transcription reaction.

The samples were centrifuged briefly and incubated in a programmed heating block (Program temperature control system, PC-700, Astec) at 37°C for 90 minutes followed by denaturation at 100°C for 15 minutes and rapid cooling on ice. The cDNA was then stored at -20°C unless it was to be used immediately in the PCR.

## 2.4 AMPLIFICATION OF cDNA BY REVERSE TRANSCRIBED POLYMERASE CHAIN REACTION (RT-PCR)

### 2.4.1 Generation of primers for the RT-PCR

As low levels of mRNA expression may be evident and the efficiency of reverse transcriptase may be very low, a technique which can increase the quantity of cDNA



target so that it may be analysed relatively easily is desirable. Such a technique is PCR. The principle of PCR is that two short regions of known sequence (primers) are used to amplify a segment of DNA which lies between them. These primers are usually oligonucleotides of around 20-24 nucleotides in length and are typically complementary to sequences that lie on opposite strands of the template DNA. The template DNA is denatured by heating in a large molar excess of both oligonucleotides with the four dNTPs and Taq DNA-dependent DNA polymerase (usually 94-95°C). The reaction mixture is then cooled to a temperature low enough that the primers will anneal to the template efficiently but high enough that they will not bind to non-specific sequences (range 50-65°C). The next stage of the PCR is to heat the mixture to around 72°C to allow extension of the annealed primers by Taq DNA polymerase. This cycle of denaturation, annealing and extension is then repeated 25-40 times, each cycle effectively doubling the quantity of desired cDNA product. The product may then be analysed by agarose gel electrophoresis and its identity confirmed by restriction enzyme digestion, Southern blotting or sequencing.

The sequences of primers for MSX-2 and its pseudogene pMSX-2 were supplied by Professor Paul Sharpe, UMDS, London. Those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and osteocalcin were supplied by Dr. Mark Birch, University of Liverpool. Those for BMP-2 and BMP-4 were derived from the sequences of the BMP clones downloaded from the Daresbury database and using the Amplify package to check primer sequences. The primers were cross-referenced to check for non-specific amplification of other members of the BMP family.

The sequences of the primers and the sizes of their PCR products are:

MSX-2:        5'-GGAGCGGCGTGGATGCAGGAACC-3' (sense)  
                 5'-TGGTGCACGCAGGGTTAGCAGCG-3' (anti-sense)

When amplified from genomic DNA, these primers produce a fragment of 3.5 kb in length (not visible on a gel as it is not readily amplifiable). From cDNA, a 584 bp fragment is produced.

osteocalcin: 5'-ATGAGAGCCCTCACACTCCTCG-3' (sense)  
                 5'-GATCTGGCCCGGCATCTTCGC-3' (anti-sense)

These primers produce a fragment of 304 bp in length from cDNA.

GAPDH:       5'-GGTGAAGGTCGGAGTCAACGG-3' (sense)  
                 5'-CCAGTACTCAGGAAGGTGCTA-3' (anti-sense)

These primers produce a fragment of 511 bp in length from cDNA.

BMP-2:        5'-CGTGACCAGACTTTTGGACACC-3' (sense)  
                 5'-TGTTTCATCTTGGTGCAAAGACCT-3' (anti-sense)

The PCR product derived from cDNA here is 205 bp in length.

BMP-4: 5'-GAGCTCCTGCGGGACTTCGAGGC-3' (sense)  
 5'-TCCAGCGAAGGACCGCAGGGCTC-3' (anti-sense)

The PCR product derived from cDNA here is 521 bp in length.

All of the above primers were supplied in concentrated stocks which were diluted to 20-30 pmoles/  $\mu$ l with water and stored in small aliquots at -20°C until use.

#### 2.4.2 Amplification of cDNA for MSX-2/pMSX-2, osteocalcin, GAPDH, BMP-2 and BMP-4

PCR for each template target was set up individually so that problems with primer dimers and with competition for dNTPS and Taq would be minimised. This also meant that visible product on a gel would not be confused with excessive quantities of template.

In setting up a PCR, it is important to minimise the risk of aerosol contamination with previously amplified material. This is done by keeping a separate set of pipettes which are dedicated for use in PCR and which do not come into contact with any DNA unless filter tips are used. A different pipette is used for loading gels and this procedure is carried out in a separate area from the location where the PCR is set up and where all stocks of reagents are aliquoted.

The reaction mix was made as a mastermix where all reagents except for the mineral oil overlay and the cDNA were placed in a single large tube and aliquoted:

2.5 $\mu$ l	10x reaction buffer
2.5 mM	MgCl <sub>2</sub>
10-15 pmoles	5' primer
10-15 pmoles	3' primer
200 $\mu$ M	dNTP mix
0.5 units	Taq polymerase
to 24 $\mu$ l	distilled water

This was then overlaid with 25  $\mu$ l mineral oil and 1  $\mu$ l cDNA was added through the oil layer. The tubes were sealed and spun briefly at 12000g before being cycled in a PCR machine (either Program Temp Control System PC-700, Astec or DNA thermal cycler version 2.2, Perkin Elmer Cetus) under the following conditions:

Initial denaturation: 94°C for 4 minutes;  
 Cycling: 95°C for 1 minute;  
 62°C for 1 minute (BMP-2 and BMP-4) or  
 65°C for 1 minute (GAPDH, MSX-2 and osteocalcin);  
 and, 72°C for 1.5 minutes.

This cycling step was carried out for 30- 40 cycles as appropriate.

Final extension: 72°C for 7 minutes;

Cooling: 4°C until analysis by gel electrophoresis.

To act as a positive control, templates for each set of primers were amplified and analysed alongside each set of samples. A water control was also set up in parallel to control for aerosol contamination with previously amplified material.

## 2.5 MOLECULAR CLONING TECHNIQUES

### 2.5.1 Transformation of *E. coli*.

To obtain plasmid in sufficient quantity for manipulation, restriction enzyme digestion or probe generation, the plasmid must be transferred into bacteria where it will increase in copy number with increase in bacteria population. Such transformation can only occur when the bacterial cells have been made competent, i.e. permeable to DNA. The bacteria (*E. coli*, strain DH5 $\alpha$ ) used in these transformations were supplied competent.

A 500  $\mu$ l stock of competent cells was thawed on ice. Aliquots of 50  $\mu$ l were placed in 0.5 ml Eppendorf tubes on ice. To each tube, 25 ng of plasmid were added. An additional tube to which 15  $\mu$ l water were added was included as a control for ampicillin resistance of the bacteria. The tubes were incubated at 0°C for 30 minutes to facilitate plasmid uptake prior to a heat shock at 42°C for 90 seconds to close the pores in the bacterial walls. Following this, 900  $\mu$ l of bacterial growth medium were added and mixed before a 45 minute incubation at 37°C. 100  $\mu$ l of the mix were then plated out onto agar containing ampicillin at a final concentration of 100  $\mu$ g/ml and incubated at 37°C for no longer than 16 hours.

Next day, a selection of colonies from agar plates was inoculated into 20 ml Sterilin tubes containing 10 ml bacterial growth medium and ampicillin and incubated at 37°C for 5-16 hours. Before inoculation of a larger volume (100- 1000 ml) of medium, it is necessary to confirm that the plasmid is present by firstly purifying a small quantity of DNA from the bacteria and then subjecting it to several restriction enzyme digestions.

### 2.5.2 Preparation of plasmid DNA from bacterial cultures

The bacteria were treated according to the Wizard Minipreps DNA purification system (Promega). 1.5 ml culture were spun down at top speed in a benchtop microcentrifuge (MSE microcentaur microfuge) for 2 minutes and the supernatant removed. This step was carried out twice more so that almost half of the bacterial culture was used. 200  $\mu$ l Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100  $\mu$ g/ml RNase A) were added to the cell pellet and the suspension was transferred to a fresh microfuge tube. 200  $\mu$ l Cell Lysis Solution were added (0.2 M NaOH, 1% SDS) and mixed by inversion until the suspension cleared. 200  $\mu$ l Neutralisation Solution were added (1.32 M potassium acetate, pH 4.8) and the mixture was inverted several times before being spun at top speed in a microfuge. The cleared supernatant was decanted into a fresh tube.

The DNA Purification Resin was thoroughly mixed prior to each removal of an aliquot. 1 ml was added to the cleared supernatant and mixed by inversion. The suspension was purified by spin column centrifugation and eluted with water. The plasmid was then stored at -20°C until use.

### 2.5.3 Restriction enzyme digestion of DNA

Restriction enzymes are endonucleases which bind specifically to and cleave double-stranded DNA at or close to a conserved sequence of usually four to six base pairs in length. This sequence is known as the recognition site and generally displays two-fold symmetry. The sequence may be cut by a restriction enzyme in two ways: either the enzyme cleaves both strands of the DNA at the axis of symmetry to generate fragments of DNA which carry blunt ends: or, it cuts each strand at similar locations on opposite sides of the axis of symmetry, generating fragments of DNA that carry overhanging ends.

This technique was used in this project to determine the identity of plasmid DNA isolated from bacterial culture by comparing the digested products with a DNA marker ladder and to cleave out an insert in a plasmid so that, following purification from either the agarose gel or from solution, it could be used to generate a radioactive probe.

Generally, 5-10 µg of plasmid were digested in 100 µl solution containing manufacturer's buffer, bovine serum albumin (BSA) and up to 50 units of enzyme. The digestion was checked after 3 hours' incubation at the required temperature by electrophoresing a 1 µl aliquot adjacent to uncut plasmid at 100 volts for 20 minutes. Completely digested product showed only bands of the expected size(s) and no background bands, which indicate undigested material. The digested band was then ready for purification by precipitation or elution from the agarose gel.

## 2.6 GEL ELECTROPHORESIS

Size fractionation, identification and purification of DNA, RNA and proteins can be carried out by electrophoresis through polyacrylamide or agarose gels. DNA or RNA was loaded into wells in a solution usually containing a Ficoll dye (5x Ficoll dye stocks are 30% Ficoll-400, 100 mM EDTA pH 7.0 and either bromophenol blue or xylene cyanol, used for agarose gels at 0.1% concentration). Appropriate size markers were run alongside DNA and RNA samples if the gel was to be used for a size fractionation purpose.

### 2.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used throughout this investigation for visualisation of PCR products, integrity of RNA and examination of restriction endonuclease digestion of plasmid DNA. Gels, unless used for Northern blot analysis

(see 2.7.2), were made up in 1x TAE (see Appendix 1) and run in this same buffer at a maximum of 120 volts. The concentration of agarose used depended on the length of the nucleic acid being examined: the optimal resolution of 0.7% agarose is 0.8-10 kb, where that of 2% agarose is 100-2000 bp (Sambrook et al, 1989a).

Nucleic acid was visualised by ethidium bromide staining and UV fluorescence at 254 nm on a UV transilluminator (Chromato-Vue Transilluminator, model M-20, Ultra-Violet Products Ltd). Photographs were taken of the gels using a hand-held camera (Polaroid Direct Screen Instant Camera, model DS34) with instant film (Polaroid Professional Coaterless Black and White Instant Pack Film 667).

#### 2.6.2 Removal of DNA fragments from agarose minigels

Wearing appropriate protection, the DNA band of interest was excised from the agarose gel using a scalpel. The piece of agarose was trimmed to remove excess gel material and placed in a preweighed Eppendorf tube. The tube plus gel segment was weighed again and the weight of agarose segment calculated (1 gram agarose equals approximately 1 ml), as 3 volumes of sodium iodide (NaI) solution were required. The tube was incubated at 55°C for 5 minutes to dissolve the agarose. An aliquot of GLASSMILK suspension was vortexed and 5 µl added for 5 µg or less of DNA (1 µl was added for every additional 500 ng DNA above 5 µg): GLASSMILK suspension contains silica particles to which the DNA adsorbs under these conditions.

The suspension was allowed to stand for 5 minutes at room temperature with vortexing every minute to keep the silica particles in suspension. The tube was then pulse-spun for 5 seconds at top speed and the supernatant was transferred to another tube (this was kept to ensure that all the DNA had been eluted). The pellet was spun again for another 5 seconds and any remaining NaI solution was removed with a pipette. 10-50 volumes NEW WASH (NaCl, ethanol, water) were added and the pellet was resuspended by digging into it with a pipette tip while pipetting up and down. The suspension was spun for 5 seconds and the supernatant discarded. This washing step was repeated twice more. The pellet was resuspended in water and incubated at 55°C for 3 minutes then centrifuged for 30 seconds to obtain a firm pellet. The supernatant containing the eluted DNA was carefully removed to a fresh tube. This elution step was repeated once more. The purified DNA was stored at -20°C until use.

#### 2.6.3 Preparation of gels for electrophoresing RNA

RNA was analysed by agarose gel electrophoresis for two reasons: to check its integrity and for the purpose of blotting it onto a membrane so that it may be probed with a fragment of radioactively labelled DNA complementary to an mRNA of interest. Throughout this type of electrophoresis, all reagents were dedicated to RNA use only and were autoclaved where possible. All plasticware and glassware was treated appropriately to ensure that equipment was RNase-free: plasticware was rinsed in

DEPC-treated water or BDH water, and glassware was baked at 180°C for 16 hours following autoclaving. Gloves were worn throughout and changed frequently.

## 2.7 GEL BLOTTING TECHNIQUES

### 2.7.1 Southern blotting

This technique was used to fix DNA onto a nylon membrane so that a fragment of radioactively labelled DNA complementary to a sequence of interest could bind to it.

Following electrophoresis and examination by UV analysis, the gel was placed back into buffer and the Southern blot apparatus was set up as follows: a glass plate was placed on an inverted support inside a tank. On this was placed a wick of 3MM paper cut to the same width as the gel. Onto this was placed 1-2 sheets of 3MM paper cut to the same dimensions as the gel. Into the tank were poured approximately 500 ml 0.4 M NaOH - sufficient so that the ends of the wick were well covered - allowing the wick and sheets of 3MM to be dampened. A fresh 10 ml pipette was rolled gently but firmly over the paper to remove any bubbles. Parafilm was used to seal the edges of the sheets of paper. The gel was removed from the buffer and placed, upside-down, onto the sheets of paper and rolled over with the pipette. A pre-cut piece of nylon membrane (Hybond N<sup>+</sup>, Amersham Life Sciences), labelled in pencil with the date and experiment, was gently laid onto the gel and rolled over. 2-3 more sheets of 3MM paper were placed on top, and 6 sheets of blotting paper (Sigma) on top of this. Another glass sheet was added and a small weight (500-750 grams) was placed on top. This was allowed to blot for 5-16 hours before disassembly, at which point the layers down to the last sheet of 3MM paper above the gel were removed. The top layer of 3MM paper below the gel was picked up and the whole gel "sandwich" was inverted. The now top layer of paper was removed and a pencil was used to mark the gel wells onto the membrane. Once this was done, the gel was disposed of and the membrane was placed in 2x SSC (see Appendix 1) for 5 minutes to remove sodium hydroxide. The membrane was allowed to dry briefly before being sealed in a plastic hybridisation bag and stored at -20°C until probed.

### 2.7.2 Northern blotting

The agarose gels for Northern blotting were made according to a protocol involving formaldehyde and formamide. 0.9% agarose was boiled in 1x MOPS (see Appendix 1) and allowed to cool slightly before 0.17 volume of formaldehyde was added in a fume hood. The gel was mixed thoroughly and poured. The gel tank was filled with 1x MOPS.

The samples were pelleted and dried in a dessicator then dissolved in 20  $\mu$ l DEPC-treated water at a concentration of 1  $\mu$ g/  $\mu$ l. To these were added:

20  $\mu$ l formamide  
 7  $\mu$ l formaldehyde  
 2  $\mu$ l bromophenol blue dye (see Appendix 1)  
 final concentration of 1x MOPS

These were denatured at 70°C for 10 minutes before being placed directly on ice for 5 minutes following which they were loaded onto the gel and electrophoresed at approximately 4 volts/ cm length.

The apparatus for Northern blotting was essentially similar to that for Southern blotting, except that the RNA was blotted in 10x SSC as compared to 0.4 M NaOH and the lower papers and membrane were all presoaked in 10x SSC. This was allowed to blot overnight then dismantled as above. In this case, the membrane was washed for 10 minutes in 50 mM sodium phosphate, pH 7.2 (diluted in DEPC-treated water prior to use from 1M stock, see Appendix 1), then it was placed in a UV crosslinker (CL-1000 UV crosslinker, UVP) for 2 minutes at 1200  $\mu$ W/ cm<sup>2</sup>. The blot was then baked at 80°C for 60 minutes to fix the RNA. To examine the integrity of the RNA, the blot was stained for 2 minutes in a solution of methylene blue (0.03% methylene blue, 0.3 M sodium acetate, pH 4.2). Excess dye was removed with repeated washes in DEPC-treated water until maximum contrast was obtained. Two strongly dyed bands representing 28S and 18S ribosomal RNA were observed indicating that RNA was intact and undegraded. The blot was then wrapped in Saranwrap and stored at -20°C until use.

## 2.8 RADIOACTIVE PROBING OF BLOTTED MEMBRANES

### 2.8.1 Preparation of the probe

Linearised purified plasmid or a fragment of DNA was labelled for use as a probe using one of two kits: the random primer labelling kit (Gibco BRL) or the Prime-a-gene labelling kit (Promega) respectively. 25-100 ng DNA were denatured by heating to 95°C for 5 minutes and then rapidly cooled by placing in ice. Using the random primer labelling kit, to this tube on ice were added, in the following order:

20 mM	dATP
20 mM	dGTP
20 mM	dTTP
7.5 $\mu$ l	random primer buffer mixture
25 $\mu$ Ci	$\alpha^{32}$ P-dCTP
10 $\mu$ l	water

For the Prime-a-gene kit, the following were added to the denatured DNA:

5 $\mu$ l	5x labelling buffer
20 $\mu$ M	dATP
20 $\mu$ M	dGTP
20 $\mu$ M	dTTP
2 $\mu$ l	acetylated bovine serum albumin (BSA)
50 $\mu$ Ci	$\alpha^{32}$ P-dCTP

to 49  $\mu$ l with water.

2x 0.5  $\mu$ l were removed for spotting onto filter paper, then 5 units Klenow DNA polymerase were added. The tube was incubated at 25°C for 60 minutes then 2x 0.5  $\mu$ l aliquots were removed for spotting. In the case of the random primer kit, 2.5  $\mu$ l STOP buffer were added to the mixture to terminate the incorporation reaction; for the Prime-a-gene kit, the mixture was heated to 95°C for 2 minutes then chilled in an ice bath prior to addition of EDTA to a final concentration of 20 mM.

The incorporation of label into the DNA was calculated using the spotted filters thus: one of each of the time-point papers was washed in 3x 0.2 M sodium phosphate, 1x water and 1x ethanol and allowed to air dry. The papers were then placed in scintillation tubes and 250  $\mu$ l scintillation fluid were added. The counts were then read by a  $\beta$ -counter (liquid scintillation counter, Rackbeta model 1209, LKB Wallac). The counts were converted to percentages to make comparisons more understandable.

Taking the counts at time=0, the percentage counts after washing as compared to those before washing gave a background level as no incorporation of label can take place without enzyme. The counts at time=60 were taken next: the percentage of counts after washing compared to those before washing give a total incorporation level, including background. Therefore, to obtain an absolute level of incorporation, the background percentage must be subtracted from the total percentage. This final figure should be at least 70% to indicate reasonable incorporation. The probe can be stored at -20°C for a couple of days or used immediately in a hybridisation reaction.

### 2.8.2 Hybridisation

Church's buffer (see Appendix 1) was heated to hybridisation temperature, then sufficient buffer to keep the membrane wet was added to it in a hybridisation bottle and placed in a rotating waterbath at 60°C for at least 3 hours. Labelled probe was added following a change of buffer. This was allowed to hybridise at 65-70°C for at least 16 hours then the buffer was removed and the membrane was monitored with a hand-held  $\beta$ -counter (series 900 mini-monitor, Mini-Instruments Ltd) for non-specific hybridisation. This was removed with repeated treatments of wash buffer (see Appendix 1) at 5 degrees below the hybridisation temperature, with the membrane being checked between each wash for background levels. If there was still a high level of background



present, the temperature at which the wash took place was increased to hybridisation temperature.

When a satisfactory level of background radioactivity was reached the blot was allowed to dry very briefly, removing only the excess of solution before being wrapped in Saranwrap. It was placed in an autoradiography cassette (Cronex Quanta Fast Detail Cassette, DuPont) with film (DuPont) and laid down at  $-70^{\circ}\text{C}$  for 1 hour initially. This film was developed and examined and the length of time of exposure of the next film against the blot was adjusted accordingly.

### 2.8.3 Stripping the blot

Following satisfactory exposure to film, the probe was removed from the blot by treatment with strip solution (see Appendix 1). The blot was treated at  $80^{\circ}\text{C}$  for 30-60 minutes, depending on the intensity of the radioactive signal. When no detectable radioactivity remained, the blot was wrapped and exposed to film for up to a week. The film was developed and examined for residual signal. In the event that signal remained the procedure was repeated until any signal remaining was suitably low.

## 2.9 ANALYSIS OF DATA

### 2.9.1 Image analysis

In order to gauge a change in the level of expression of mRNA, some form of quantitation is required. In these experiments, GAPDH was used as a housekeeping gene, insofar as its expression does not appear to be affected by treatment of the cells with any of the reagents used. The intensity of signal obtained following blotting and probing of GAPDH RT-PCR products should therefore reflect the quantity of total RNA, which should be equal in all lanes if the same number of cells were used or if the same amount of template was introduced. Any variation in the GAPDH band intensities must be removed to allow analysis of the band intensities of the other genes of interest, as variation here could simply be due to differences in the number of cells, and therefore in the absolute quantity of RNA originally in the sample, and not because of a true effect of treatment with the factors.

Image analysis allows an image of the autoradiograph or gel to be captured into a computer where variations in background staining and band intensity are removed, and where measurements of the intensities can be made for later comparison.

The gel was placed on a UV transilluminator and the image was photographed (TM-765 Kinetic Pulnix camera with cosmicar TV zoom lens). It was opened in the Imagedok program (Kinetic Imaging Ltd.) on a Hewlett Packard Vectra VL2 computer, where the contrast was adjusted to obtain the clearest image. In the case of analysing an autoradiograph, visible light was used and the same process continued. A hard copy of the image was produced when required (UP-D860E digital graphic printer, Sony). The

image was saved as a TIFF file onto a disk in the SyQuest drive (EZ 135MB drive, SyQuest) and opened in the Phoretix program (Kinetic Imaging Ltd.).

Here, the image was handled as follows: the first step was that a lane profile had to be created. The image contained one row of samples consisting of DNA amplified using one set of primers (in this example, for BMP-4, see figure 2.2). The row was selected by clicking in one corner and dragging the rectangle over the entire row. This was now termed a "lane". The process was repeated for another "lane" containing samples amplified with primers for GAPDH.

The next stage was to define the peaks and edges of each sample, then to remove the background from the "lane" (see figure 2.2). This was achieved by opening the "analyse lanes" option, then the "remove background" option. The following stage was to measure the intensity of the detected bands. This could be viewed as a table of absolute measurements. These data were saved onto the SyQuest drive and opened into the Microsoft Excel program (Microsoft Corporation Inc.) for further analysis.

To simplify, only the first two sample bands from figure 2.2 were analysed for the following example (figure 2.3). The intensity measurement of each GAPDH band was divided by that of a designated GAPDH control, usually that derived from an untreated sample (table i, figure 2.3). This analysis was also performed for BMP-4 (table ii, figure 2.3). The final stage was to remove inter-sample variation in loading by expressing each BMP-4 value as a ratio of the related GAPDH value (table iii, figure 2.3). In this example, it can be seen that the true ratio of BMP-4 expression between untreated and treated samples is higher than it would be were the variation not removed, thus reflecting a more accurate effect of treatment on gene expression.

### 2.9.2 Statistical analysis

In order to examine whether the effects of agents on osteoblast-related gene expression were statistically significant, the data were subject to analysis. It could not be assumed that the data were normally distributed. Therefore the Kruskal-Wallis test was selected as the most suitable statistical analysis test for these investigations as it is a non-parametric test, and therefore does not assume a normal distribution of data. It can be used to analyse more than three sample groups of varying sizes and, as it is based on a ranking system and compares the median values of the samples, the absolute magnitudes of the changes in gene expression seen in these experiments is not considered, merely whether or not a change was observed following treatment with one or more agents.

The H value is used to compare the median values between all the sample groups tested. A value determined experimentally shown to be equal to or greater than the critical H value indicates a statistically significant result (Neave, 1995). It is calculated by the formula:

$$H = \frac{12}{N(N+1)} \sum_{k=1}^k (R^2/n) - 3(N+1)$$

where N= total number of values analysed, k= number of samples,  $\Sigma$ = sum of, R= rank of each value, n= size of each sample (number of repeats per treatment). This is illustrated in the example below (Neave, 1995) where samples of the mileages per gallon for three different engine designs are compared:

design	mileage per gallon				ranks				rank sums
a	19.8	20.5	20.8	19.7	4	6	7.5	2.5	20
b	21.7	20.8	21.2		10	7.5	9		26.5
c	19.7	19.4	19.9		2.5	1	5		8.5

$$H = [12/10(11)] \times (20^2/4 + 26.5^2/3 + 8.5^2/3) - 3(11) = 6.073$$

The critical H value for three samples of unequal sizes of 4, 3 and 3 is 5.791, therefore there is a significant difference in performance between the three designs of engine. This does not describe between which samples the significant difference lies.

The second stage of the Kruskal-Wallis test is to compare every sample with each of the other samples by use of Q values. These determine the statistical significance of the differences observed between individual sample medians and, as for H values, an experimentally determined value equal to or greater than the critical Q value is indicative of statistical significance (Heath, 1995). The Q value is calculated by the formula below:

$$Q = \frac{R_1 - R_2}{\text{square root } [N(N+1)/12 \times (1/n_1 + 1/n_2)]}$$

where R= mean rank of sample, N= total numbers of values analysed in Kruskal-Wallis test, n= size of each sample (number of repeats per treatment). In the above example, three tests are required: a compared to b, then compared to c, then b compared to c. Working through the first of these:

$$Q = \frac{26.5/3 - 20/4}{\text{square root } [10(11)/12 \times (1/4 + 1/3)]} = \frac{8.833 - 5}{\text{square root}(9.167 \times 0.583)} = \frac{3.833}{2.312} = 1.658$$

The critical value for Q in this instance is 2.394, therefore there is no significant difference between the two engine designs, a and b.

a		
b		1.658
c	<u>7.281</u>	<u>4.973</u>
	c	b
		a

The other values showing comparison of a and c or b and c are shown to be significant, therefore the engine design c gives significantly lower mileage than the other designs. The groups arranged on the vertical axis are compared to those on the horizontal axis, such that a value denoting a significant decrease (underlined) means that the expression level of the group on the vertical axis is significantly lower than that of the group on the horizontal axis to which it is being compared.

It is to be noted however that testing for the value of Q was only performed where a statistically significant H value was obtained: in those instances where H was not seen to be significant (see Appendix 3), it was not appropriate to calculate the Q values.

## 2.10 GENERAL TECHNIQUES

### 2.10.1 Extraction with phenol:chloroform

The method most frequently used to remove proteins from aqueous solution is to extract with phenol:chloroform, as two different organic solvents are more efficient than one, and then again with chloroform, to remove trace residues of phenol. The phenol was supplied as equilibrated with Tris-HCl pH 8.0. An equal volume of phenol:(chloroform:isoamyl alcohol= 24:1), that is, phenol:chloroform:isoamyl alcohol = 25:24:1, was added to a tube containing impure nucleic acid and the contents were mixed to form an emulsion. The tube was then centrifuged at 12000g for 5 minutes and the upper aqueous phase, containing the nucleic acids, was transferred to a fresh tube. An equal volume of chloroform was added and mixed and centrifuged as above. The nucleic acids were recovered by precipitation with ethanol in the case of DNA or isopropanol in the case of RNA.

### 2.10.2 Precipitation of nucleic acids

Precipitation using either ethanol or isopropanol is the most widely used method of concentrating nucleic acids. For the recovery of small amounts of low molecular weight nucleic acids, the aqueous solution of nucleic acid was added to an equal volume of chilled isopropanol and mixed by inversion. The nucleic acid precipitated out of solution after being left at -70°C overnight (RNA) or at -20°C for between 30 minutes and 16 hours (fragments of digested DNA). Precipitation of undigested plasmid DNA was in double volume of ethanol at -20°C overnight.

### 2.10.3 Quantitation of nucleic acids

Depending on the purity of the sample, two methods were used to estimate the concentration of nucleic acid in solution. If the sample was very pure, optical density measurements of the amount of ultra-violet light absorbed by the nucleotide bases was carried out. If there were significant amounts of contaminants such as phenol, proteins,

proteoglycans, agarose or other nucleic acids, estimation of the quantity of nucleic acid was done by analysis on agarose minigels.

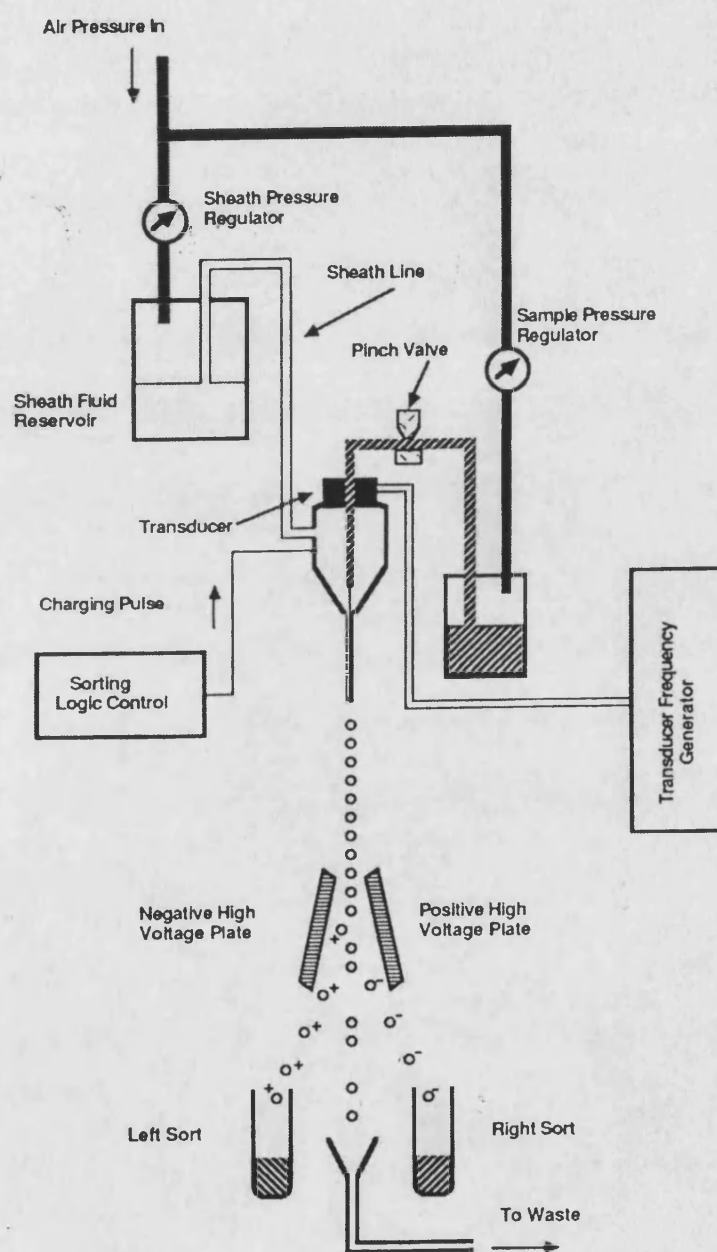
#### 2.10.3.1 Spectrophotometric measurement

Readings were taken at 260 nm and 280 nm: the reading at 260 nm allows calculation of the amount of nucleic acid present, as optical density (OD) measurement of 1 corresponds to approximately 50 µg/ ml double-stranded DNA, 40 µg/ ml for single-stranded DNA and RNA, and 20 µg/ ml for oligonucleotides. The ratio between OD<sub>260</sub> and OD<sub>280</sub> gives an estimate of the purity of the nucleic acid sample (pure preparations of DNA and RNA give values of 1.8 and 2.0 respectively) (Sambrook et al, 1989b). Contamination with phenol or protein reduces this value.

#### 2.10.3.2 Minigel method

If there is a possibility that the nucleic acid preparation contains contaminants, electrophoresis through agarose minigels is rapid and convenient. By comparing the intensity of fluorescence of ethidium bromide-stained bands of nucleic acid of unknown concentration with that of standards of known concentration run side by side, the quantity of nucleic acid in the test sample can be estimated.

Figure 2.1 Fluorescence activated cell sorting (Carter and Meyer, 1990)



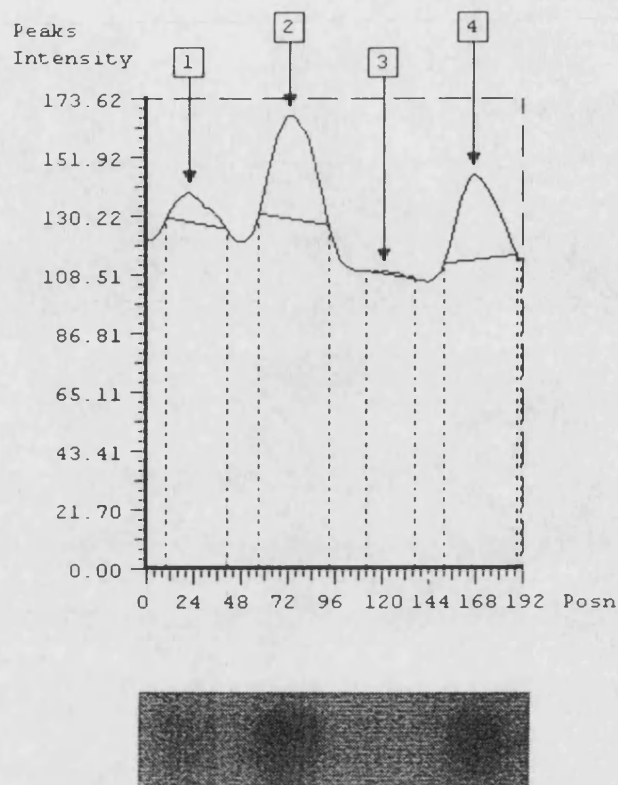


Figure 2.2 Densitometric analysis of RT-PCR gels or Southern blots

In this example, a lane was captured from a blot of BMP-4 RT-PCR product and subjected to analysis by densitometry. On the plot above, maximal densities (peaks) are indicated by arrows and the edges of the sample bands by dotted lines. Background was removed also (indicated by lines joining the highest point of the dotted edges). Therefore, the volumes measured in this analysis are represented by the remaining areas under the curves. Clearly, when compared to bands 1 and 3, the bands in 2 and 4 are more dense.

Figure 2.3 Example of calculation to determine the ratio of mRNA expression

BMP-4 expression in untreated and treated cells

i	Sample	GAPDH measurement	ratio cf control	
	control	36084	1	
	treated	9046.5	0.25	
ii	Sample	BMP-4 measurement	ratio cf control	
	control	14212.5	1	
	treated	43873.5	3.09	
iii	Sample	ratio cf GAPDH control	ratio cf BMP-4 control	adjusted ratio cf BMP-4 control
	control	1	1	1
	treated	0.25	3.09	12

Figure 2.3 Calculation of ratio of BMP-4 mRNA expression after treatment with calcitriol

The actual measurements of GAPDH are expressed as a ratio of the control sample (see i). The same is done for the BMP-4 measurements (see ii). Finally, the differences in GAPDH must be taken into account, therefore the ratios of BMP-4 mRNA transcripts are expressed relative to the ratio of GAPDH in the same treatment group (see iii).

2



## **Chapter 3**

**Altered pattern of gene expression in osteoblast-like cells following exposure to calcitriol and members of the TGF-beta superfamily**

## Introduction

A sequence of gene expression in osteoblasts has been elucidated: the expression levels of genes characteristic of proliferation and extracellular matrix biosynthesis, such as c-fos, c-myc, histone-4, type I collagen, fibronectin and TGF-beta, are transiently upregulated in the first ten days of culture of primary rat osteoblasts. As proliferation decreases, expression of these genes is attenuated and genes associated with the development, maturation and organisation of the extracellular matrix are transcribed, including alkaline phosphatase and matrix gla protein. The expression of these genes is downregulated coincident with the upregulation of that of genes thought to influence extracellular matrix mineralisation, for example osteopontin and osteocalcin (Owen et al; Aronow et al, 1990).

Studies with osteoblast-like cell lines indicate a more de-regulated progression from proliferation to differentiation: proliferation of the cells continues, albeit at a gradually reduced rate, as they become committed to a differentiated phenotype, resulting in the expression of mineralisation genes in the absence of an extracellular matrix (Stein et al, 1990). This suggests that the mechanisms which control the progression from proliferation to matrix maturation and then mineralisation are de-regulated in these cells. Therefore, osteoblastic cell lines are potentially useful tools in the study of bone formation to elucidate the mechanisms whereby osteoblastic proliferation and differentiation are controlled. Several cell lines now exist which mimic osteoblast characteristics at different stages of differentiation and maturation, as evidenced by their gene expression patterns.

MG-63 cells (CRL 1423, American Tissue Culture Collection) are derived from an osteosarcoma removed from a 14 year-old male and are distinguished by a high proliferation rate. Previous studies with the MG-63 line have indicated that they share characteristics with osteoblastic cells: they express basal and inducible alkaline phosphatase and osteocalcin and they respond to treatment with calcitriol (Mahonen et al, 1990; Szulc and Delmas, 1996). Unstimulated MG-63 cells thus mimic an immature stage of osteoblast differentiation. The cells attach well to fibronectin and vitronectin and produce fibroblastic growth factors-1 and -2 (FGF-1 and FGF-2) but, unlike osteoblasts, MG-63 cells do not express platelet-derived growth factor (PDGF), nor do they produce a mineralised extracellular matrix (Hauschka et al, 1988).

Rodent cells have been studied extensively as putative models of osteoblastic function. MC3T3-E1 cells are derived from mouse calvariae and require an ascorbic acid-dependent extracellular matrix for expression of the osteoblastic phenotype (Franceschi and Iyer, 1992). ROS 17/2.8 cells are derived from a rat osteosarcoma and represent a mature osteoblast phenotype (Williams et al, 1994): expression of alkaline phosphatase mRNA peaks during active cell growth and large amounts of osteocalcin are produced constitutively (Rodan et al, 1988).

The pattern of MSX-2, BMP-2, BMP-4 and osteocalcin gene expression was studied in osteoblast-like cells exposed to a variety of growth factors and agents which might modulate the expression of these genes. RNA extracted from cells was analysed by RT-PCR and Southern blotting and/ or Northern blotting. For each, densitometric analysis of the autoradiographs was used to determine treatment-induced changes in mRNA transcript expression. The "housekeeping gene" glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to correct for treatment-induced variations in cell number and/ or differences in the quantity of mRNA/ cDNA template. All values were normalised using the level of expression of the housekeeping gene as an internal standard, and compared to the level of gene expression in the untreated control.

### 3.1 Establishing optimum conditions for the polymerase chain reaction

#### Magnesium chloride concentration

Magnesium chloride is an essential chelating agent in the polymerase chain reaction, insufficient or too great a quantity resulting in a reduction in the efficiency of the polymerase enzyme (Guide to Optimising PCR, Promega). To gauge the optimal concentration of magnesium chloride, a titration was performed using concentrations of  $Mg^{2+}$  between 0.5 mM and 5 mM in increments of 0.5 mM, using equivalent amounts of MSX-2 cDNA plasmid as a template (see figure 3.1a). The optimal concentration for the MSX-2 PCR was shown to be 2.5 mM magnesium chloride. All other parameters for the PCR were gauged at this magnesium chloride concentration.

#### Annealing temperature

According to the ratio of G/C to A/T residues in the primer sequences selected in the PCR, the annealing temperature of the primer to the target sequence of the cDNA will vary: the greater the ratio of G/C to A/T, the higher the annealing temperature (Sambrook et al, 1989). Using equivalent amounts of cDNA template and 2.5 mM magnesium chloride, reaction mixtures with primers specific for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH were cycled for 35 cycles at annealing temperatures ranging from 55 °C to 68 °C. Based on the results of this series of PCRs (see figure 3.1b), the annealing temperature for BMP-2 and BMP-4 was selected to be 62 °C (lane 3); that for MSX-2, osteocalcin and GAPDH was 65 °C (lane 4).

#### Sensitivity of the PCR

False negative signals can arise due to a concentration of target template which falls below the threshold of sensitivity of the PCR. Therefore the sensitivity of this reaction was gauged using MSX-2 template diluted serially 1 in 10 in the range 1 µg to 1 fg using conditions as previously described for 35 cycles (figure 3.1c). The threshold of sensitivity was shown to lie between 10 pg and 1 pg.

#### Cycle number

Using an excessive number of cycles can result in saturation of the signal and an inability to detect a change in the level of template expression. Therefore it was

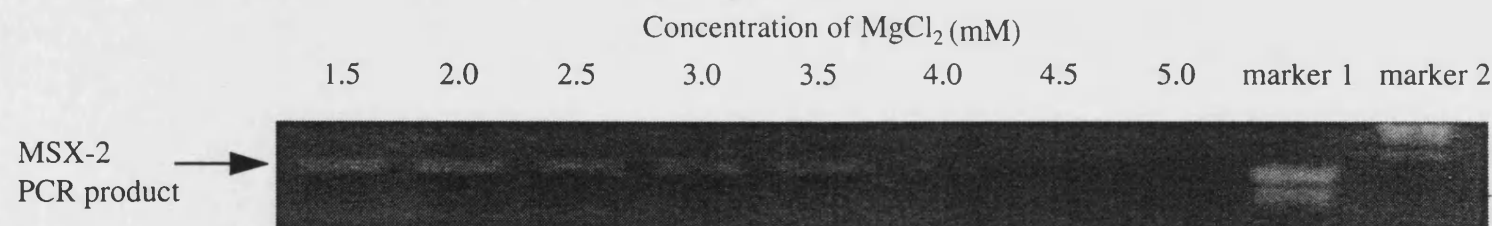


Figure 3.1a Optimisation of the magnesium chloride concentration in the polymerase chain reaction (PCR)

Magnesium chloride was added to reaction tubes prior to the addition of Taq polymerase and MSX-2 cDNA-containing plasmid template to give final concentrations ranging from 1.5- 5 mM. The reaction mixes were amplified for 35 cycles. Products were analysed in a 1.5% agarose gel in TAE buffer run at 80 V for 90 minutes.

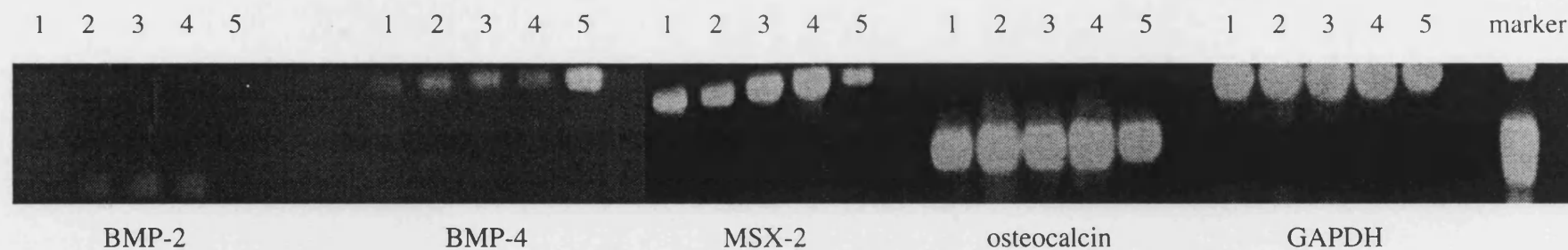


Figure 3.1b Optimisation of the annealing temperatures for each primer set in the PCR

Using the optimum concentration of magnesium chloride as previously determined, equivalent amounts of cDNA template were amplified for 35 cycles at annealing temperatures of 55, 60, 62, 65 and 68 °C (lanes 1 to 5 respectively). Following analysis by agarose gel electrophoresis, the temperature at which the brightest band was produced was selected as optimal and used in future analyses.

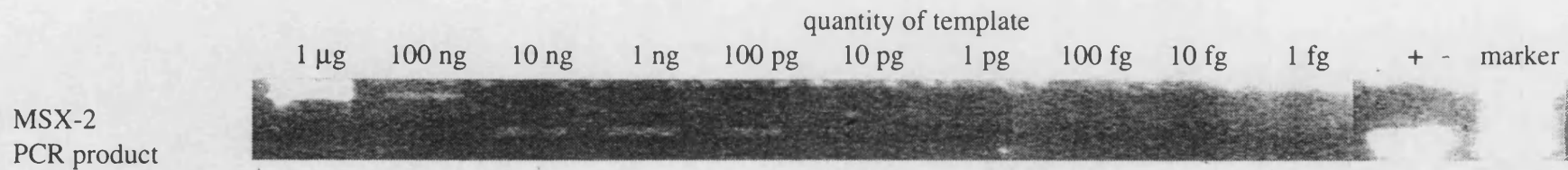


Figure 3.1c Titration of DNA template to assess the sensitivity of the PCR

MSX-2 cDNA was serially diluted in increments of 10 and applied to the reaction mixes. PCR was performed for 35 cycles and the products analysed by agarose gel electrophoresis. At higher concentrations of the template (1 µg and 100 ng, lanes 1 and 2), a band of a larger size than the expected 584 bp was seen. The lack of interference and the appearance of a band of the expected size at lower concentrations of template (10 ng and lower) suggests that the higher band may have been caused by mispriming as a result of a high concentration of salt or another contaminant in the plasmid stock. The signal band was seen to disappear at concentrations of plasmid equivalent to 1 picogram (pg) or lower.

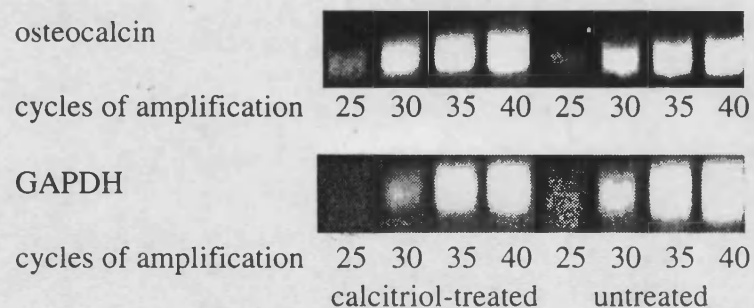


Figure 3.1d Optimising the numbers of cycles required for amplification of templates for GAPDH and osteocalcin PCRs

MG-63 cells were cultured in DMEM containing 10% fetal calf serum until confluency, at which point DMEM containing 2% fetal calf serum was added to the cells. After 24 hours, the cells were exposed to 1 nM calcitriol for 24 hours, the RNA was harvested and RT-PCR and agarose gel electrophoresis performed. The figure above illustrates the differences in band intensity following increasing numbers of cycles during the PCR for osteocalcin and GAPDH. At higher numbers of cycles, the difference in intensity between bands is reduced as the reaction becomes saturated: any apparent variation in gene expression is abolished. The contrast between osteocalcin mRNA expression in control and calcitriol-treated cultures is greatest at low cycle numbers. Therefore, amplification of 30-35 cycles was used as standard in future analysis, and adjusted accordingly thereafter for more accurate comparison.

necessary perform a cycle titration (figure 3.1d). PCRs for osteocalcin and GAPDH using cycle numbers in the range 25 to 40 cycles were performed: at 25 cycles, the difference in osteocalcin expression between untreated and calcitriol-treated cells is clear; at 30 cycles or greater, this difference in osteocalcin expression becomes difficult to discern. Therefore in each following experiment, depending on the quantity of RNA extracted, 30- 35 cycles was used as an initial cycle run and if no signal or a saturated signal was the result, a second run was performed with an appropriately adjusted number of cycles.

### 3.2 Effects of calcitriol treatment on osteocalcin mRNA transcript expression

Osteocalcin expression is directly affected by calcitriol due to the presence of a vitamin D response element (VDRE) in the promoter region of the gene (Breen et al, 1994, b; reviewed by Lian and Stein, 1992). Expression of this gene was examined following treatment of MG-63 cells for 24 hours with various doses of calcitriol (see figure 3.2). RNA was extracted and subjected to RT-PCR followed by Southern blotting for osteocalcin and GAPDH. Steady state levels of osteocalcin were unaffected by treatment with  $10^{-11}$  M calcitriol, but at doses greater than this ( $10^{-7}$  M to  $10^{-10}$  M) a substantial increase in osteocalcin mRNA was observed when compared with untreated control. Interestingly, a similar profile for the expression of osteocalcin protein was seen following treatment with calcitriol (Beresford et al, 1984).

### 3.3 Effects of members of the TGF-beta superfamily, alone or in combination with calcitriol, on the expression of osteoblast-related genes in MG-63 cells

TGF-beta-1 and BMPs are distantly related members of the TGF-beta superfamily and signal intracellularly via cell surface serine/threonine kinase receptors. This experiment was designed to compare the effects of these agents on the transcription of the same genes in MG-63 cells, and to examine the modulatory effects of calcitriol on these factors (see figure 3.3). Confluent MG-63 cells maintained in DMEM supplemented with 10% fetal calf serum were cultured for 24 hours in DMEM containing 2% fetal calf serum prior to the addition of the following concentrations of growth factors: 1- 100 nM calcitriol, 1- 250 ng/ ml BMP-2/ BMP-7, 1- 25 ng/ ml BMP-4, 1- 50 ng/ ml BMP-6 and 0.05- 1.25 ng/ ml TGF-beta. Also examined were the effects of addition of 10 nM calcitriol to these doses of factors. After exposure to the agents for 24 hours, RNA was extracted and analysed for mRNA expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting. Densitometric analysis of the blots was performed: all values are relative to untreated control and to the levels of GAPDH and therefore to loading. The results from three independent experiments of dose responses for calcitriol are shown in table 3.1a. A dose response for members of the TGF-beta superfamily, alone and in combination with 10 nM calcitriol, is summarised in table 3.1b. The results from three independent

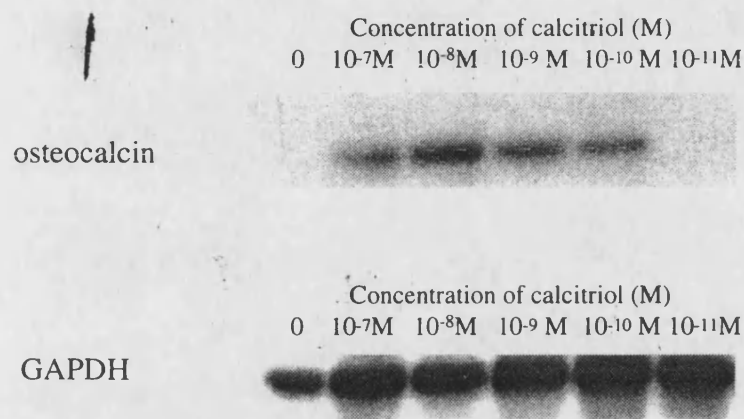


Fig. 3.2 Dose-dependent increase in osteocalcin mRNA expression in MG-63 cells following treatment with calcitriol

MG-63 cells were cultured in DMEM containing 10% fetal calf serum until 24 hours prior to treatment with calcitriol, when the serum concentration in the replacement medium was dropped to 2%. After 24 hours, RNA was harvested and RT-PCR with primers specific for GAPDH and osteocalcin was performed, followed by Southern blotting with probes for these genes. PCR was performed for 30 cycles. This experiment was carried out twice ( $n=2$ ).

experiments where selected doses of TGF-beta superfamily members and of calcitriol were added alone or in combination are shown in table 3.1c.

#### Effects of calcitriol alone

Taking differences in GAPDH levels and therefore loading into account, expression of BMP-4, MSX-2 and osteocalcin is increased by calcitriol, with maximal levels occurring at a dose of calcitriol of 10 nM, when compared with control (table 3.1a). A biphasic effect of the agent on the expression of MSX-2 and BMP-4 transcripts was shown: MSX-2 and BMP-4 mRNA expression at 100 nM calcitriol being less than at 10 nM, although still greater than control. Overall, there was a modest increase in BMP-2 mRNA expression following treatment with calcitriol.

#### Effects of BMP-2 alone

At all doses tested, BMP-2 increased the expression of its own mRNA, with maximal effects occurring at 1 ng/ ml BMP-2 (table 3.1b). At the lowest dose tested (1 ng/ ml), BMP-2 also had a stimulatory effect on the mRNA expression of osteocalcin and BMP-4. At higher doses however, the effect became less emphatic (expression of osteocalcin transcripts) or even inhibitory (BMP-4 mRNA expression). MSX-2 mRNA expression was increased at higher doses of the agent to levels twice that of untreated control.

#### Effects of BMP-2 and calcitriol in combination

When compared with calcitriol treatment alone, BMP-2 antagonised the stimulatory effect of calcitriol on the level of expression of the mRNAs studied (table 3.1c). When compared with the equivalent dose of BMP-2 alone, the effects of combining the agent with calcitriol were more complex: in two out of three repeats, MSX-2, osteocalcin and BMP-4 mRNA expression levels were increased, and BMP-2 mRNA levels were decreased (table 3.1b).

#### BMP-4 alone

When compared with control, the doses of BMP-4 tested had no effect on the expression of the mRNAs for osteocalcin or BMP-4 (table 3.1b). There was, however, a modest increase in the level of expression of MSX-2 and BMP-2 transcripts, with maximal increases observed at 10 ng/ ml and 25 ng/ ml BMP-4 respectively.

#### BMP-4 and calcitriol in combination

Similar to the effects of BMP-2 in combination with calcitriol when compared to treatment with calcitriol alone, all doses tested of BMP-4 antagonised the stimulatory effects of 10 nM calcitriol on the expression of the four genes studied (table 3.1b). Treatment with BMP-4 and calcitriol in combination stimulated BMP-4 mRNA expression only at the maximal dose when compared to treatment with BMP-4 alone. BMP-2 mRNA levels were decreased when compared to the effects of BMP-4 alone, and MSX-2 expression was stimulated. Osteocalcin expression was maximally induced at the lowest BMP-4 dose (1 ng/ ml BMP-4 plus 10 nM calcitriol) and is dose-



dependently decreased thereafter (10 ng/ ml and 25 ng/ ml BMP-4 plus 10 nM calcitriol) to levels still greater than treatment with BMP-4 alone.

#### Effects of BMP-6 alone

When compared with control, at all doses the addition of BMP-6 increased the expression of all of the genes studied by 2- to 7-fold (table 3.1b).

#### Effects of BMP-6 and calcitriol in combination

Compared to treatment with either agent alone, the effects of BMP-6 and calcitriol in combination were to decrease BMP-2 expression (table 3.1b). In contrast, the expression levels of MSX-2, osteocalcin and BMP-4 transcripts were essentially unchanged or increased following treatment with both factors in combination when compared with treatment with either factor alone.

#### BMP-7 alone

Treatment with BMP-7 had a substantial positive effect on the expression of all of the genes studied (tables 3.1b and 3.1c). For MSX-2, the maximal increase was observed at 50 ng/ ml BMP-7, whereas for osteocalcin, BMP-2 and BMP-4, this occurred at the 10 ng/ ml dose (table 3.1b).

#### BMP-7 and calcitriol in combination

When added in combination, calcitriol and BMP-7 interacted positively to regulate the expression of transcripts for MSX-2, osteocalcin and BMP-4 (50, 10 and 50, and 1- 250 ng/ ml respectively, tables 3.1b and 3.1c). In contrast, treatment with both factors in combination stimulated BMP-2 transcript expression to a lesser extent than treatment with calcitriol alone (table 3.1b).

#### Effects of TGF-beta-1

TGF-beta-1 stimulated expression of all genes tested when compared to untreated control (table 3.1b). Maximal increase was seen with the lowest dose (0.05 ng/ ml) for BMP-4 and MSX-2, and with a dose of 0.25 ng/ ml for BMP-2. With increased dose of TGF-beta-1, this stimulation of expression was lessened but not reduced below 2-fold the level of the untreated control. Osteocalcin expression levels were stimulated to 4-fold those of steady state, an effect which was maintained regardless of dose.

#### Effects of TGF-beta-1 in combination with calcitriol

TGF-beta or calcitriol alone stimulate the levels of mRNA transcript of all of the genes studied (tables 3.1a and 3.1b). However, the changes in transcript expression following treatment of MG-63 cells with TGF-beta and calcitriol in combination were extremely variable from one experiment to another and therefore are difficult to interpret (tables 3.1b and 3.1c). It is possible that the conflicting signals of proliferation (TGF-beta) and differentiation (calcitriol) may be resulting in unpredictable consequences for gene expression. It would be appropriate therefore to investigate the effects of different doses of agents in combination on the transcript levels of the genes of interest.

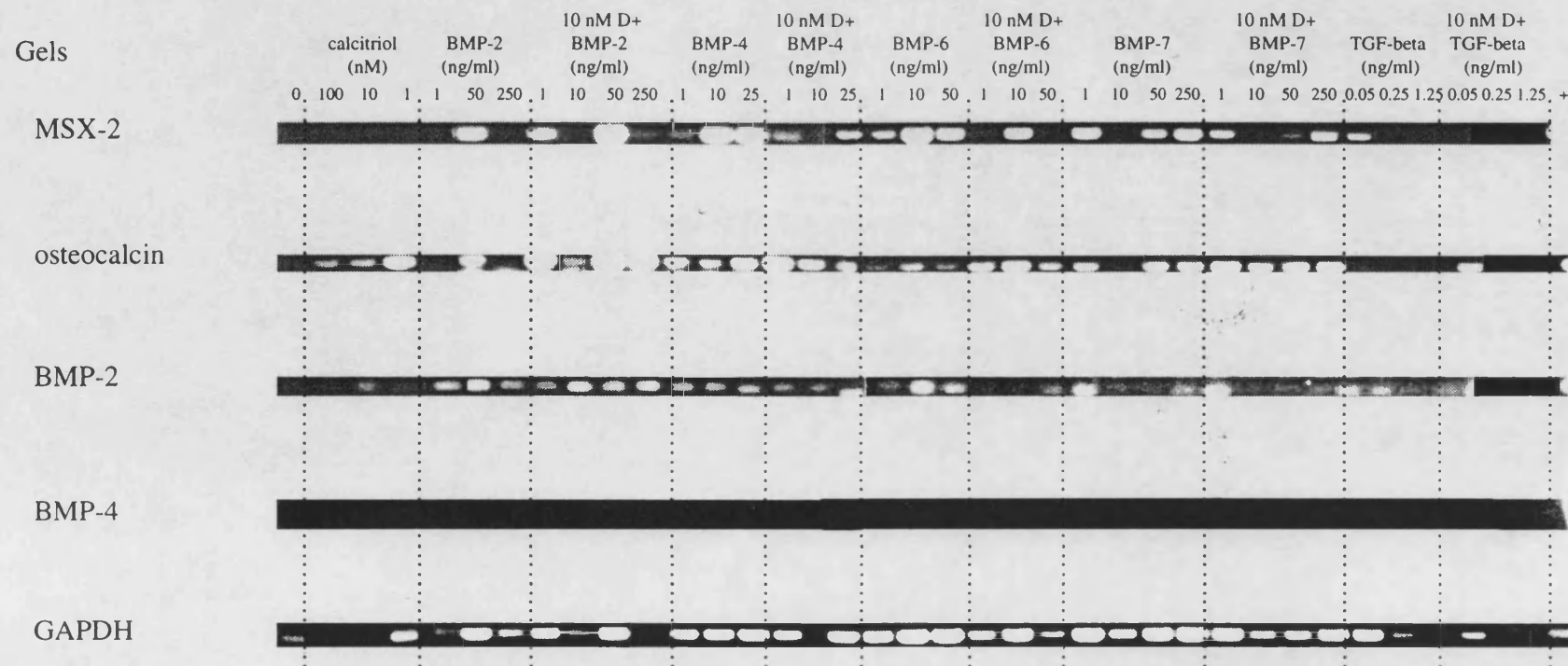


Figure 3.3a Effects of members of the TGF-beta superfamily (+/- calcitriol) on the expression of osteoblast-related genes in MG-63 cells

MG-63 cells were cultured in DMEM containing 10% fetal calf serum until confluent, at which point this was replaced with DMEM containing 2% fetal calf serum. After 24 hours, the cells were exposed for 24 hours to ranges of doses of the agents as indicated in the above figure: 1- 100 nM calcitriol, 1-250 ng/ ml BMP-2/ BMP-7, 1-25 ng/ ml BMP-4, 1-50 ng/ ml BMP-6 and 0.05- 1.25 ng/ ml TGF-beta. Also examined were the effects of the addition of 10 nM calcitriol to increasing doses of the agents (as indicated above by "D"). RNA was harvested and subjected to RT-PCR with primers specific for MSX-2 (35 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (40 cycles) and GAPDH (30 cycles). The products were analysed by agarose gel electrophoresis. This figure is representative of two independent experiments.

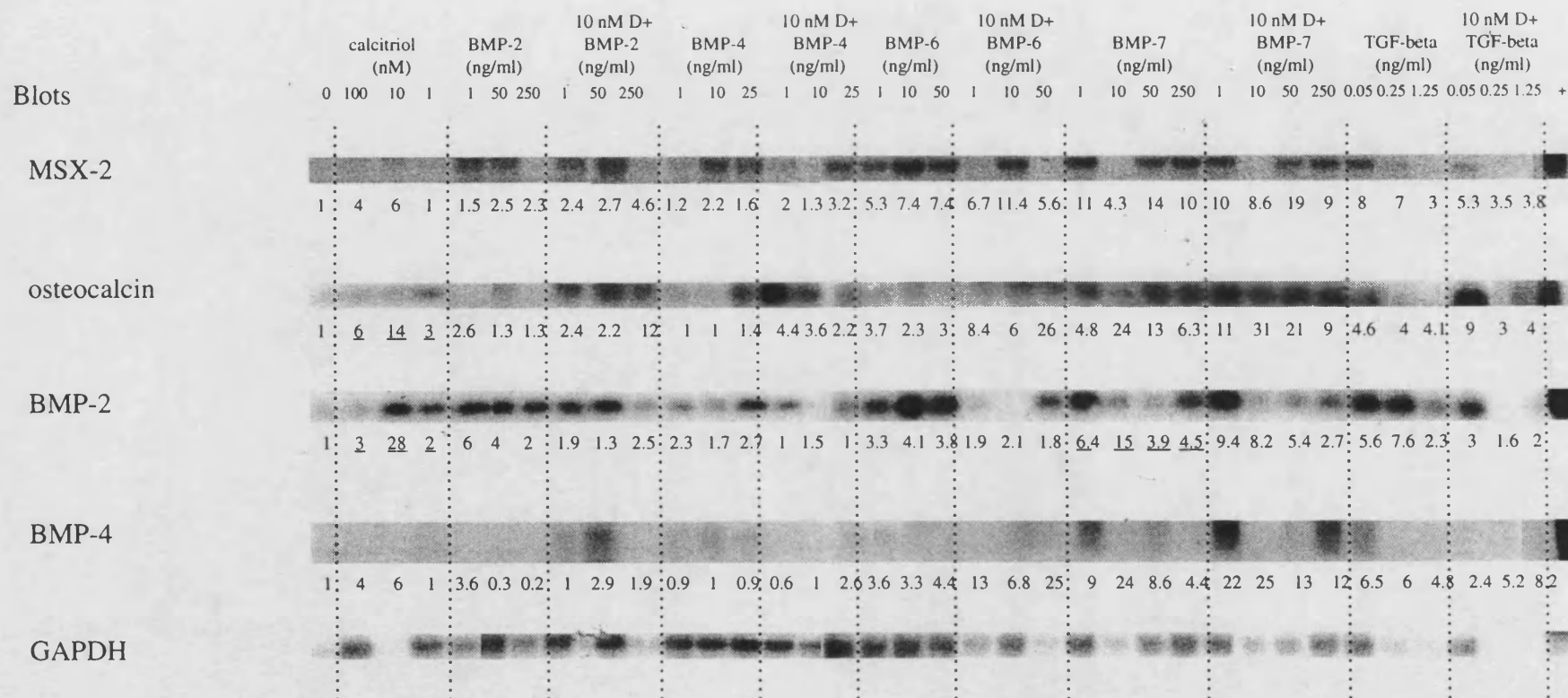


Figure 3.3b Effects of members of the TGF-beta superfamily (+/- calcitriol) on the expression of osteoblast-related genes in MG-63 cells

This figure shows the Southern blots of the RT-PCR products analysed by agarose gel electrophoresis in figure 3.3a. These blots are representative of those derived from two independent experiments. The values under each blot are derived from densitometric analysis and are relative to control and to GAPDH levels. Underlined values show statistical significant H values.

mRNA	calcitriol (nM)			
	0	1	10	100
MSX-2	1	1	6	4
	1	0	6	nd
	1	2	2	nd
osteocalcin	1	3	14	6
	1	1	2	nd
	1	3	3	nd
BMP-2	1	2	28	3
	1	1	1	nd
	1	2	1	nd
BMP-4	1	1	6	4
	1	1	3	nd
	1	2	4	nd

Table 3.1a The effects of calcitriol on the expression of osteoblast-related genes in MG-63 cells

Confluent MG-63 cells maintained in DMEM supplemented with 10% fetal calf serum were cultured in DMEM containing 2% fetal calf serum 24 hours before the addition of calcitriol in the range 1- 100 nM. Following exposure to the factor for 24 hours, RNA was extracted and analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2 BMP-4 and GAPDH transcript expression. The table above is a summary of the densitometric values obtained from three independent experiments. nd, not done.

Factor		None	BMP-2			BMP-4			BMP-6			BMP-7				TGF-beta		
Dose (ng/ ml)			1	50	250	1	10	25	1	10	50	1	10	50	250	0.05	0.25	1.25
mRNA	10 nM calcitriol																	
	-	1	1.5	2.5	2.3	1.2	2.2	1.6	5.3	7.4	7.4	10.8	4.3	14.2	10.2	8	7	3
MSX-2	+	6	2.4	2.7	4.6	2	1.3	3.2	6.7	11.4	5.6	10	8.6	18.6	9	5.3	3.5	3.8
	-	1	2.6	1.3	1.3	1	1	1.4	3.7	2.3	3	4.8	24	13	6.3	4.6	4	4.1
osteocalcin	+	14	2.4	2.2	12	4.4	3.6	2.2	8.4	6	26	11	31	21	9	9	3	4
	-	1	6	4	2	2.3	1.7	2.7	3.3	4.1	3.8	6.4	15.2	3.9	4.5	5.6	7.6	2.3
BMP-2	+	28	1.9	1.3	2.5	1	1.5	1	1.9	2.1	1.8	9.4	8.2	5.4	2.7	3	1.6	2
	-	1	3.6	0.3	0.2	0.9	1	0.9	3.6	3.3	4.4	9	24	8.6	4.4	6.5	6	4.8
BMP-4	+	6	1	2.9	1.9	0.6	1	2.6	12.5	6.8	25	22	25	13	12	2.4	5.2	8.2

Table 3.1b The effects of members of the TGF-beta superfamily, alone or in combination with 10 nM calcitriol, on the expression of osteoblast-related genes in MG-63 cells

Confluent MG-63 cells maintained in DMEM supplemented with 10% fetal calf serum were cultured in DMEM containing 2% fetal calf serum 24 hours prior to exposure for 24 hours to the agents above. RNA was extracted and analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH mRNA expression. The table above is a summary of the densitometric values obtained from one experiment.

Factor		50 ng/ ml BMP-2			50 ng/ ml BMP-7			50 pg/ ml TGF-beta			10nM calcitriol		
mRNA	10 nM calcitriol												
	-	3	2	36	14	3	3	8	3	67	6	6	2
MSX-2	+	3	3	160	19	7	327	5	5	119	nd	nd	nd
	-	1	1	2	13	3	2	5	2	1	14	2	3
osteocalcin	+	2	3	4	21	4	7	9	2	5	nd	nd	nd
	-	4	2	2	4	2	2	6	1	0	28	1	1
BMP-2	+	1	0.5	2	5	4	10	3	1	4	nd	nd	nd
	-	0	0	0	9	1	0	7	1	1	6	3	4
BMP-4	+	3	1	1	13	3	11	2	2	4	nd	nd	nd

Table 3.1c The effects of selected doses of calcitriol and members of the TGF-beta superfamily, alone or in combination, on the expression of osteoblast-related genes in MG-63 cells

Confluent MG-63 cells maintained in DMEM supplemented with 10% fetal calf serum were cultured in DMEM containing 2% fetal calf serum 24 hours prior to the addition of the above factors. After 24 hours' exposure, RNA was extracted and analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH transcript expression. The table above is a summary of the densitometric values obtained from three independent experiments. nd, not done.

Data from this experiment were pooled (tables 3.1a-3.1c) for the purposes of statistical analysis, which can be seen in Appendix 3, null hypotheses 1-7.

### 3.4 The effects of interactions between calcitriol and members of the TGF-beta superfamily on the expression of osteoblast-related genes in MG-63 cells

Following on from the last experiment in which the interactions between calcitriol and members of the TGF-beta superfamily had marked effects on the expression of osteoblast-related gene transcripts in MG-63 cells, the interactions of these agents were examined further in three experiments (one example shown in figure 3.4). Where possible, doses of the factors were selected so that the effects of combinations of agents could be observed without those of one agent obscuring those of another: these were 1 nM calcitriol, 50 ng/ ml BMP-2 or BMP-7 and 0.05 ng/ ml TGF-beta-1. The results of the densitometric analysis for three independent experiments are shown in table 3.2a, and were subjected to statistical analysis (see Appendix 3). In the following section, emphasis has been placed on those combinations of factors that yielded similar trends, in terms of direction, but not necessarily of magnitude, in all three experiments.

As shown previously (table 3.1a), treatment with 1 nM calcitriol alone increased the level of expression of transcripts for osteocalcin and BMP-2, but did not have a consistent effect on that of MSX-2 or BMP-4. Treatment with BMP-2 increased the level of expression of its own mRNA and, in two of three experiments, that of MSX-2 (table 3.2a). That this trend is representative of the true action of this factor on the expression of MSX-2 in this cell line is supported by the results of previous experiments (table 3.1b). Treatment with BMP-2 also consistently decreased the level of BMP-4 mRNA expression. When added in combination, calcitriol antagonised the stimulatory effect of BMP-2 on the expression of MSX-2, but for osteocalcin and BMP-2 transcript expression, the results obtained were similar to those seen following treatment with calcitriol alone (table 3.2a). BMP-4 mRNA expression was at a similar level following treatment with calcitriol and BMP-2 in combination to that seen after treatment with BMP-2 alone.

When added alone, 50 ng/ ml BMP-7 increased the level of transcript expression for MSX-2, osteocalcin and BMP-2, but had no consistent effect on the expression of BMP-4 mRNA (table 3.2a). When added in combination, calcitriol had a slight antagonistic effect on the stimulation of expression of transcripts for MSX-2, osteocalcin and BMP-4 compared to those seen following treatment with BMP-7 alone, and had an essentially additive effect on the expression of BMP-2 mRNA.

Treatment with TGF-beta markedly increased the level of expression of MSX-2 transcripts (3- 67-fold) and also increased the level of expression of osteocalcin transcripts. In contrast, TGF-beta had no consistent effect upon the expression of transcripts for BMP-2 or BMP-4. When added in combination, calcitriol antagonised

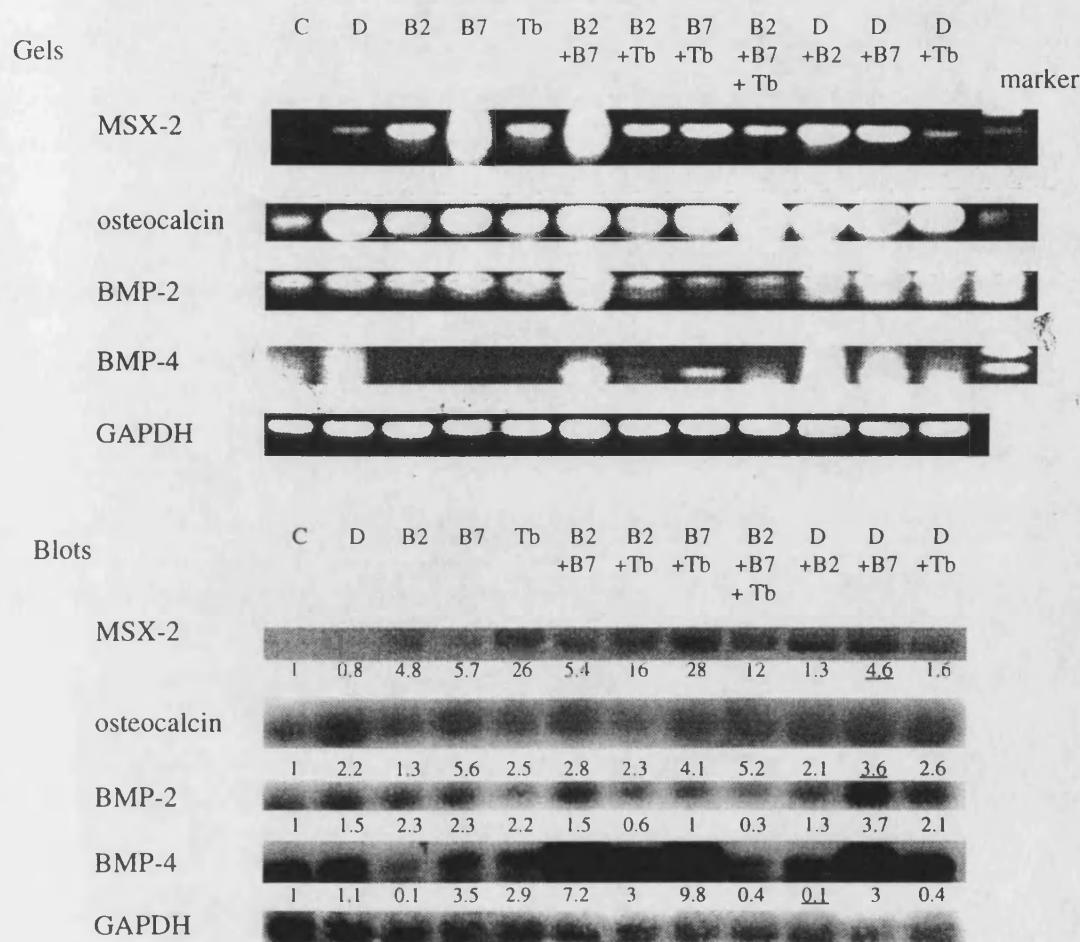


Figure 3.4 The effects of interactions between calcitriol and members of the TGF-beta superfamily on the expression of osteoblast-related genes in MG-63 cells

MG-63 cells were cultured in DMEM containing 10% fetal calf serum until 24 hours prior to treatment when this was replaced with DMEM containing 2% fetal calf serum. The cells were then exposed for 24 hours to 1 nM calcitriol (D), 50 ng/ml BMP-2 (B2), 50 ng/ml BMP-7 (B7) and 50 pg/ml TGF-beta (Tb), alone or in combination. Suboptimal doses of agents were selected where possible to examine the interactive effects on gene expression. RT-PCR was performed for MSX-2 (35 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (35 cycles) and GAPDH (30 cycles).

Apparent differences were observed between the quantity of RT-PCR product visualised by ethidium bromide staining in the gels and that seen following Southern blot analysis, for example, MSX-2 expression after treatment with BMP-7 alone. As these PCRs were carried out at a suboptimal annealing temperature of 55 °C, mispriming with non-target sequences may have occurred. However, specific probes were used under stringent hybridisation conditions (65 °C) in the Southern blotting process, therefore changes in mRNA expression observed in the Southern blotting technique were analysed by densitometry. These gels and blots are representative of three independent experiments. Values below blots are average densitometric values, with underlines showing statistical significance for H values in the Kruskal-Wallis test.



Factor	None	1 nM calcitriol	1 nM calcitriol	BMP-2	BMP-7	TGF-beta
mRNA						
MSX-2	1	0.8 (0.1-1.6)	-	4.8 (0.5-11.3)	5.7 (2.8-14.2)	26 (3.3-66.5)
			+	1.3 (0.8-1.8)	4.6 (0.8-7.5)	1.6 (0.1-2.8)
osteocalcin	1	2.2 (1.4-2.8)	-	1.3 (0.9-1.8)	5.6 (1.8-12.6)	2.5 (1.2-4.8)
			+	2.1 (1.5-2.8)	3.6 (1.8-5.9)	2.6 (1.4-3.8)
BMP-2	1	1.5 (1.2-1.9)	-	2.3 (1.5-3.6)	2.4 (1.5-3.9)	2.2 (0.1-5.6)
			+	1.3 (0.8-1.9)	3.7 (0.6-7.2)	2.1 (0.1-3.7)
BMP-4	1	1.1 (0.5-1.6)	-	0.1 (0-0.2)	3.5 (0.9-8.6)	2.9 (1.0-6.5)
			+	0.1 (0-0.4)	3 (0.4-6.8)	0.4 (0-0.7)

Table 3.2 a The effects of suboptimal doses of calcitriol, alone or in combination with members of the TGF-beta superfamily, on the expression of osteoblast-related genes in MG-63 cells

MG-63 cells maintained in DMEM supplemented with 10% fetal calf serum were cultured for 24 hours in DMEM containing 2% fetal calf serum. They were then exposed to the following concentrations of agents for 24 hours: 1 nM calcitriol, 50 ng/ ml BMP-2 or BMP-7 and 50 pg/ ml TGF-beta. RNA was extracted and analysed for the transcript expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting. Densitometric analysis was performed on the blots. Values are the average from three independent experiments with the range of values shown in parentheses.

Factor	None	BMP-2	BMP-7	TGF-beta	BMP-2 + BMP-7	BMP-2 + TGF-beta	BMP-7 + TGF-beta	BMP-2 + BMP-7 + TGF-beta
mRNA								
MSX-2	1	4.8 (0.5-11.3)	5.7 (2.8-14.2)	26 (3.3-66.5)	5.4	16.2	27.6	12
osteocalcin	1	1.3 (0.9-1.8)	5.6 (1.8-12.6)	2.5 (1.2-4.8)	2.8	2.3	4.1	5.2
BMP-2	1	2.3 (1.5-3.6)	2.4 (1.5-3.9)	2.2 (0.1-5.6)	1.5	0.6	1	0.3
BMP-4	1	0.1 (0-0.2)	3.5 (0.9-8.6)	2.9 (1.0-6.5)	7.2	3.0	9.8	0.4

Table 3.2b The effects of suboptimal doses of members of the TGF-beta superfamily, alone or in combination, on the expression of osteoblast-related genes in MG-63 cells

MG-63 cells maintained in DMEM supplemented with 10% fetal calf serum were cultured for 24 hours in DMEM containing 2% fetal calf serum. They were then exposed to the following concentrations of agents for 24 hours: 1 nM calcitriol, 50 ng/ ml BMP-2 or BMP-7 and 0.05 ng/ ml TGF-beta. RNA was analysed by RT-PCR and Southern blotting for expression of transcripts of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH. Densitometric analysis was performed on the blots. Values are the average from three independent experiments (single treatments) with the range shown in parentheses or from one experiment (combinations).

the stimulatory effect of TGF-beta on MSX-2 mRNA expression (table 3.2a). When compared with TGF-beta alone, treatment with calcitriol in combination did not alter the expression of transcripts for osteocalcin or BMP-2, but did consistently decrease the level of those for BMP-4.

In a single experiment, the effects of adding members of the TGF-beta superfamily in combination were investigated. The most striking observation was that when added in combination with BMP-7, the effects of any other family members on the level of expression of BMP-4, MSX-2 and osteocalcin transcripts were greater than those seen with these other members alone, with the most potent combination in the experiment being BMP-7 and TGF-beta (table 3.2b). BMP-2 mRNA expression was decreased to levels lower than those seen with single agents when any combination of TGF-beta superfamily members was applied to the cells (table 3.2b). When all three family members were added in combination, the level of BMP-4 and BMP-2 mRNA expression was reduced when compared with that seen in untreated cultures.

Statistical analysis for this experiment can be seen in Appendix 3, null hypotheses 8- 11.

### 3.5 The effects of calcitriol on osteoblast-related gene expression in osteoblast-like cells

MG-63 cells, MC3T3-E1 cells and ROS 17/ 2.8 cells were treated with 10 nM calcitriol for 24 hours prior to harvesting of their RNA. These cell lines were selected as models of different stages of osteoblast-like maturation, with MC3T3-E1 cells being thought to be representative of preosteoblasts (Franceschi and Iyer, 1992), MG-63 cells representing a more differentiated osteoblastic phenotype (reviewed by Aubin et al, 1993) and ROS 17/ 2.8 cells having many of the properties of mature osteoblasts (Rodan et al, 1988). Therefore, it may be possible to discern different patterns of gene expression in these different cell types. RNA was examined by Northern blot analysis for the expression of MSX-2 or *Msx-2*, alkaline phosphatase and osteocalcin. The levels of expression were normalised for loading by comparison with the methylene blue stained membranes (see table 3.3). Figure 3.5 illustrates one example of this experiment.

When analysed by Northern blot, MSX-2 expression in MG-63 cells was inhibited by calcitriol treatment when compared with control whereas osteocalcin mRNA levels were modestly increased. Calcitriol had little effect on the steady state levels of *Msx-2* in MC3T3-E1 cells, whereas in ROS 17/ 2.8 cells, *Msx-2* expression was doubled, alkaline phosphatase levels were modestly increased and osteocalcin mRNA expression was markedly increased when compared with control. Only MSX-2/ *Msx-2* expression levels were subjected to statistical analysis as insufficient repeats for the other genes were performed (see Appendix 3, null hypothesis 12).

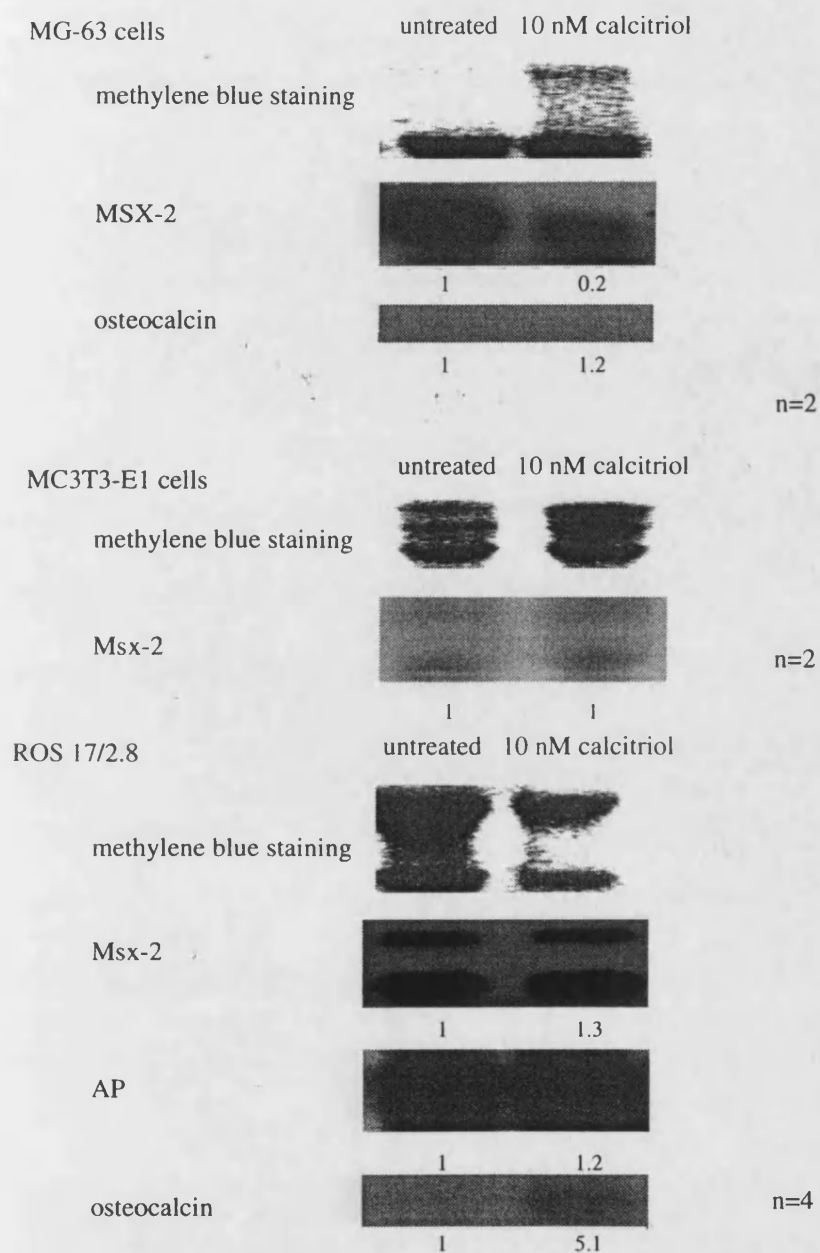


Figure 3.5 Effects of calcitriol on gene expression in osteoblast-like cells

Cells were cultured to confluency in DMEM containing 10% fetal calf serum at which point medium was replaced with DMEM containing 2% fetal calf serum. MC3T3-E1 cells were cultured in the constant presence of 50  $\mu$ M ascorbate-2-phosphate. 24 hours after replacement of the medium, cells were exposed to the agents as above for 24 hours. RNA was extracted and analysed by Northern blot. The figures shown are representative and the number of independent repeats is indicated by the value of "n". Densitometric values are shown beneath each blot.

Cell type		MG-63 cells	MC3T3-E1 cells	ROS 17/ 2.8 cells
mRNA	10 nM calcitriol			
MSX-2/Msx-2	-	1	1	1
	+	0.2 (0.1-0.4)	1.0 (0.8-1.3)	1.3 (1.0-2.1)
osteocalcin	-	1	nd	1
	+	1.2	nd	5.1
alkaline phosphatase	-	nd	nd	1
	+	nd	nd	1.2 (1.2-1.3)

Table 3.3 The effects of calcitriol on osteoblast-related gene expression in osteoblast-like cell lines as assessed by Northern blot analysis

Cells were maintained in DMEM supplemented with 10% fetal calf serum until confluent, at which point medium was replaced with DMEM containing 2% fetal calf serum. MC3T3-E1 cells were constantly cultured in the presence of 50  $\mu$ M ascorbate-2-phosphate. After 24 hours, cells were exposed to 10 nM calcitriol for a further 24 hours prior to extraction of RNA and analysis by Northern blot.

This table is a summary of up to four independent experiments: blotting for Msx-2 from ROS 17/ 2.8 cells was performed four times; that for MSX-2/Msx-2 from MG-63 and MC3T3-E1 cells, and for alkaline phosphatase for ROS 17/ 2.8 cells was performed twice; all other blotting was carried out once. Values are averaged with the range shown in parentheses and represent the relative levels of osteoblast-related gene expression in MG-63 cells, MC3T3-E1 cells and ROS 17/ 2.8 cells following treatment with 10 nM calcitriol for 24 hours. nd, not done.

### 3.6 Northern blot analysis of RNA extracted from MG-63 cells treated with calcitriol, cycloheximide and actinomycin D, alone or in combination

In order to gain some insight into the mechanisms of the regulation of expression of MSX-2, cycloheximide, a protein synthesis inhibitor, and actinomycin D, an RNA synthesis inhibitor, alone or in combination with 10 nM calcitriol, were introduced to MG-63 cells for 24 hours (figure 3.6). MG-63 cells maintained in DMEM supplemented with 10% fetal calf serum were cultured for 24 hours in DMEM containing 2% fetal calf serum. The cells were then exposed to 2.5 µg/ ml cycloheximide or 250 ng/ ml actinomycin D, alone or in combination with 10 nM calcitriol, for 24 hours prior to the harvesting of RNA and analysis for the expression of MSX-2 by Northern blot analysis. Densitometric analysis on the blot was performed and normalised using the average intensity of the two ribosomal bands (28S and 18S) following methylene blue staining (see table 3.4).

In a single experiment, a decrease in MSX-2 expression was seen following treatment with calcitriol when analysed by Northern blot as previously observed (table 3.3). When compared to untreated control, actinomycin D treatment resulted in an abrogation of MSX-2 mRNA transcript levels. Cycloheximide treatment alone resulted in increased MSX-2 expression when compared to untreated control.

Adding the agents in combination resulted in an increase in MSX-2 expression following treatment with cycloheximide and calcitriol when compared with control: MSX-2 transcript levels were stimulated when compared to treatment with calcitriol and decreased when compared to cycloheximide alone. Actinomycin D and calcitriol together inhibited MSX-2 mRNA expression when compared with control: when compared to the actions of either agent alone, a small increase in MSX-2 mRNA levels was seen.

### 3.7 Examination of the effects of over-expression of MSX-2 on calcitriol-induced MSX-2 expression in ROS 17/ 2.8 cells

ROS 17/ 2.8 cells were transfected with a human MSX-2 construct by Jane Hodgkinson, UMDS, London, using a lipofectamine protocol. Transfectants (denoted R2/8 and R2/11) were cultured continuously in G418 to prevent reversion and treated with 10 nM calcitriol for 24 hours prior to harvest of their RNA and assessment by Northern blot analysis (figure 3.7). As for the previous experiment, blots were normalised for loading using the methylene blue stained membranes. The RNAs were blotted twice and the mean of the densitometric values was used in comparison with levels in untransfected ROS 17/ 2.8, MG-63 and MC3T3-E1 cells (see table 3.5).

Following treatment of these transfectants with calcitriol, R2/11 levels were approximately halved whereas R2/8 levels of MSX-2 were doubled. Statistical analysis of the results of this experiment can be seen in Appendix 3, null hypothesis 12.

methylene blue stain

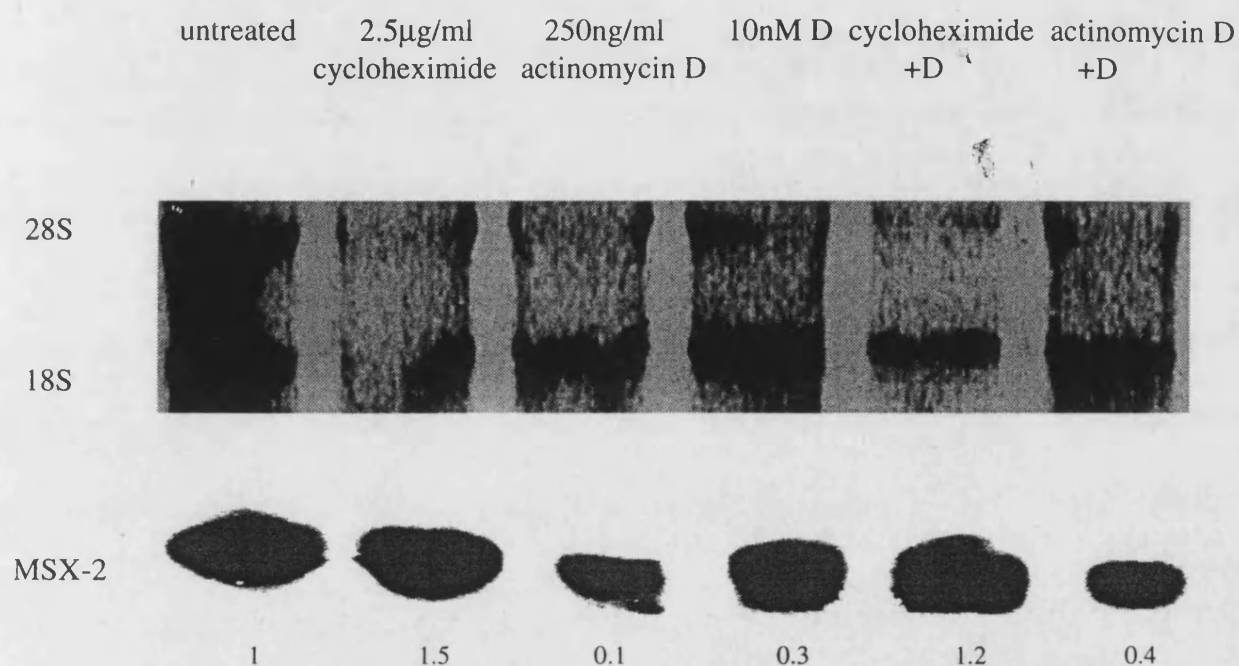


Figure 3.6 Northern blot analysis of RNA extracted from MG-63 cells treated with calcitriol, cycloheximide and actinomycin D, alone or in combination

MG-63 cells were cultured to confluency in DMEM containing 10% fetal calf serum which was replaced with DMEM containing 2% fetal calf serum 24 hours before treatment with the agents above. To gain some insight into the mechanism(s) whereby MSX-2 expression is regulated in osteoblast-like cells, confluent MG-63 cells were treated with the protein synthesis inhibitor, cycloheximide, and the RNA synthesis inhibitor, actinomycin D, alone or in combination with 10nM calcitriol (D). RNA was harvested after exposure to these agents for 24 hours and assessed by Northern blot analysis for MSX-2 mRNA transcript expression. Densitometric values are shown beneath the blot. This experiment was performed once.

	untreated	2.5 µg/ml cycloheximide	250ng/ml actinomycin D	10 nM calcitriol	10 nM calcitriol + 2.5 µg/ml cycloheximide	10 nM calcitriol + 250ng/ml actinomycin D
MSX-2	1	1.5	0.1	0.3	1.2	0.4

Table 3.4 Northern blot analysis of RNA extracted from MG-63 cells treated with calcitriol, cycloheximide and actinomycin D, alone or in combination

MG-63 cells were maintained in DMEM supplemented with 10% fetal calf serum until confluence at which point medium was replaced with DMEM containing 2% fetal calf serum. After 24 hours, the cells were exposed for 24 hours to the following doses of agents, alone or in combination: 10 nM calcitriol, 2.5 µg/ml cycloheximide and 250 ng/ml actinomycin D. RNA was extracted and subjected to analysis by Northern blot. Autoradiographs were quantitated by densitometric analysis: the table above represents the relative expression levels of MSX-2 following treatment with the agents.



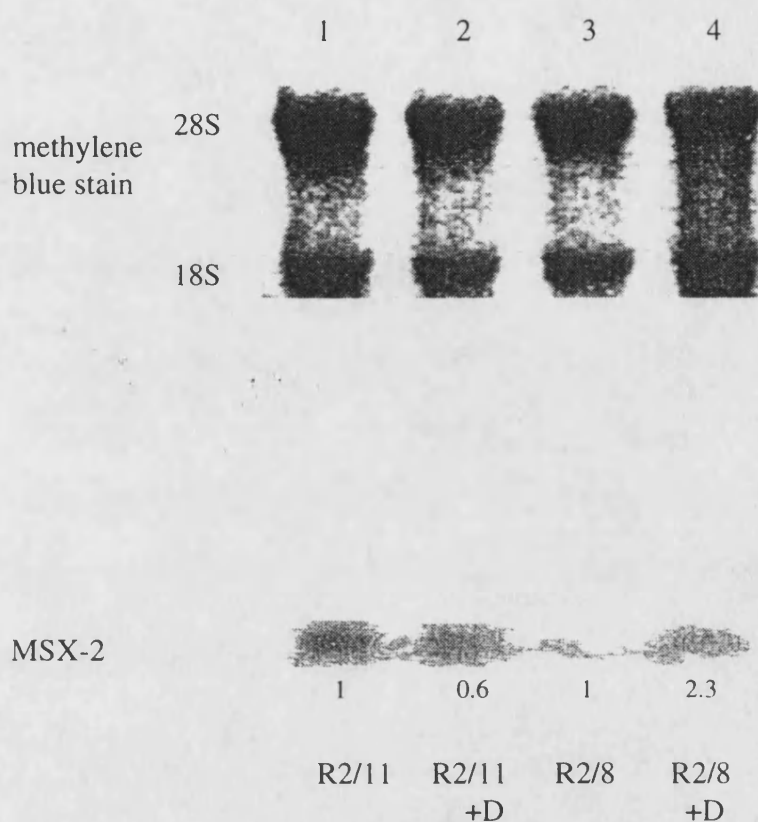


Figure 3.7 Northern blot analysis of RNA extracted from ROS 17/2.8 cells stably overexpressing MSX-2 and treated with calcitriol

Samples of total RNA derived from ROS 17/2.8 cells transfected with a construct containing the coding sequence of the human MSX-2 gene were supplied by Jane Hodgkinson, UMDS, London. Two transfectants, denoted R2/8 and R2/11, had been cultured constantly in DMEM containing G418 and treated for 24 hours with 10 nM calcitriol prior to the harvest of the RNA. This was analysed by Northern blot for the expression of MSX-2 mRNA transcripts. RNA was blotted twice. Densitometric values are shown beneath the blot.

Cell type		MG-63 (n=2)	MC3T3-E1 (n=2)	ROS 17/ 2.8 (n=4)	R2/8 (r=2)	R2/11 (r=2)
	10 nM calcitriol					
	-	1	1	1	1	1
MSX-2/msx-2	+	0.2 (0.1-0.4)	1.0 (0.8-1.3)	1.3 (1.0-2.1)	2.3 (2.1-2.5)	0.6 (0.4-0.7)

Table 3.5 The effects of over-expression of MSX-2 on calcitriol-induced expression of MSX-2 in osteoblast-like cells

Cells were maintained in DMEM supplemented with 10% fetal calf serum until confluent at which point medium was replaced with DMEM containing 2% fetal calf serum. MC3T3-E1 cells were cultured constantly in the presence of 50  $\mu$ M ascorbate-2-phosphate. After 24 hours, cells were exposed to 10 nM calcitriol for 24 hours, then RNA was extracted and analysed by Northern blot.

The table above summarises the results of a number of independent experiments, as indicated by “n”, and of repeat blotting, as denoted by “r”, and represents the relative levels of MSX-2 mRNA transcripts in different cell lines, some overexpressing MSX-2, and treated with 10nM calcitriol.

## Discussion

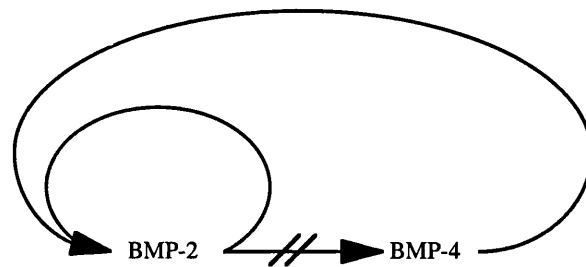
The RT-PCR technique, used extensively in the course of this research, is useful in the detection of absolute changes in gene expression, for example the absence or presence of an mRNA transcript. In order to observe more quantitative changes in gene expression, such as increases or decreases in the expression levels of mRNA transcripts elicited by treatment with an agent, an internal quantitative control would be required. Another method, such as dot-blot hybridisation/ Northern blotting, where variations in messenger mRNA levels for different genes within the same sample and between different samples run simultaneously can be quantitatively evaluated, would in hindsight be preferable. However, even with the limitations of the RT-PCR technique, consistent trends were seen in the course of the research in this project. In this discussion, and those which follow, the focus is on the changes in direction, and not in magnitude, in the expression of transcripts of osteoblast-related genes.

The investigation of gene expression in established osteoblast-like cell lines and how it differs from that of normal osteoblastic cells (herein defined as cells cultured from normal adult human bone and studied in primary culture or at first passage) may lead to some understanding of the genetic pathways that regulate osteogenic differentiation. Examination of the cascade of gene expression in osteoblast-like cells may reveal similarities with that in bone-derived cells and may support the use of these cell lines as models for osteoblast differentiation.

The importance of BMP-2 and BMP-4 in bone formation has already been discussed (see section 1.8). In this investigation, the regulation of their expression in MG-63 cells by other members of the TGF-beta superfamily and by calcitriol was shown to differ. BMP-2 mRNA levels were stimulated by calcitriol, BMP-6, BMP-7 and TGF-beta, the latter in contrast to the study in subcultures of fetal rat calvarial osteoblasts where BMP-2 expression was suppressed by TGF-beta treatment, where a higher dose (2 ng/ml) was used (Harris et al, 1994). Interestingly, BMP-2 transcript expression was upregulated by BMP-4 treatment also. The finding that BMP-2 upregulates its own expression in MG-63 cells supports the discovery that BMP-2 stimulates production of its own mRNA in rodent cells (Ghosh-Choudhury et al, 1996). This effect was maximal at the lowest dose and its magnitude decreased with increased levels of agent, therefore it would appear that in the absence of another agent, BMP-2 regulates its own expression, maintaining steady levels. High levels of BMP-2 serve to inhibit the expression of BMP-4, which was stimulated only by the lowest dose of BMP-2. The induction of BMP-2 expression by BMP-4 acts as a negative regulator of BMP-4 expression due to the inhibitory effects of higher doses of BMP-2 on BMP-4 expression.

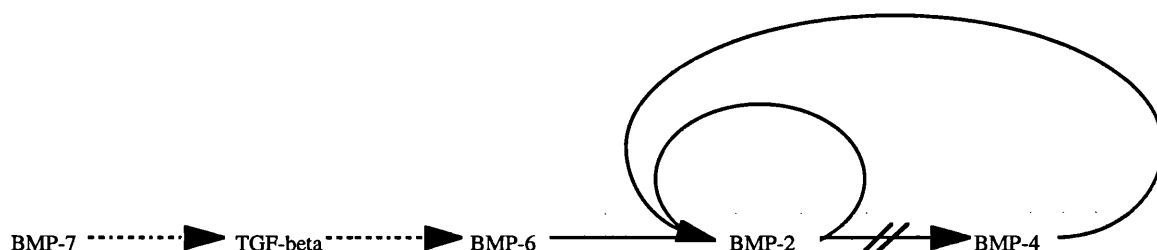
Taken together this suggests that increased levels of expression of BMP-2 augments BMP-2 expression and downregulates that of BMP-4. BMP-2 mRNA expression is stimulated by the presence of BMP-2, therefore BMP-2 expression is

maintained. Only when levels of BMP-2 decrease does expression of BMP-4 transcripts increase. This complementarity of BMP-2 and BMP-4 in vitro is reflected in development of the embryo where BMP-2 and BMP-4 respectively exhibit dorsalising and ventralising functions in insects and mammals (Rusch and Levine, 1994; Staehling-Hampton et al, 1994; Steinbeisser et al, 1995).



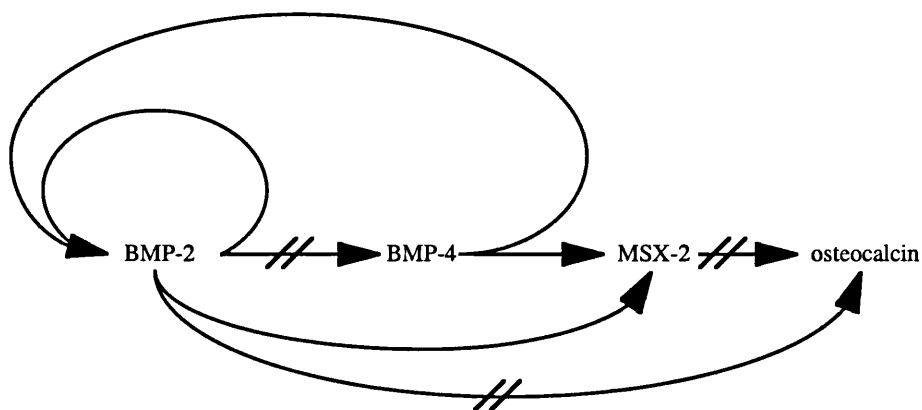
A possible model for the expression of BMP-2 and BMP-4 in human osteosarcoma cells

Also noted were the effects of BMP-4 on the expression of osteoblast-related genes where it was shown that of all the agents tested in MG-63 cells, BMP-4 was the least potent stimulator of transcript expression for any of the genes examined. For the other family members, the rank order of potency for increasing transcript expression (low to high) was BMP-2, BMP-6, TGF-beta and BMP-7. BMP-4 mRNA expression was stimulated only by the higher doses of calcitriol and was suppressed at lower concentrations of the agent. BMP-4 had no effect on its own transcription and was stimulated by BMP-6: maximal expression of BMP-4 occurred with high doses of BMP-6, suggesting that expression of BMP-6 must be upregulated in order for BMP-4 to be expressed. The trend of the effects of BMP-7 and TGF-beta on BMP-4 transcript expression indicate that these agents have little effect on the expression of this gene when compared to that in untreated cells, but stimulate the expression of BMP-2. A longer exposure to these agents would be necessary to determine their effects on BMP-4 expression as modulated by increased BMP-2 expression. Taken together, these results indicate that the actions of BMP-7 and TGF-beta occur earliest in the sequence of expression, as induction of all genes of interest followed treatment with these agents, and that a possible model of the cascade of BMP gene expression can be suggested.



A possible model for the cascade of BMP gene expression in human osteosarcoma cells

In the progression of MG-63 cells towards osteoblastic differentiation, BMP-4 and BMP-2 are expressed prior to MSX-2 and osteocalcin. High levels of BMP-2 block any increase in BMP-4 and osteocalcin mRNA levels, the effects on the latter perhaps being mediated by the induction of MSX-2 expression. BMP-4 also induces MSX-2 expression. Low levels of BMP-2 have no real effect on MSX-2 expression, but increase that of BMP-4 and galvanise differentiation by stimulating osteocalcin expression. Therefore, in the absence of a strong differentiation stimulus, BMP-2 blocks osteogenic differentiation. Moreover, it would appear that in order for osteoblastic differentiation to proceed in MG-63 cells, expression of BMP-2 must be downregulated. Thus a possible model for the cascade of osteoblast-related gene expression and of osteogenic differentiation in MG-63 osteosarcoma cells can be suggested:



A possible model for the cascade of osteoblast-related gene expression in human osteosarcoma cells

The role of calcitriol in the promotion of osteogenic differentiation has been established in several studies (Beresford et al, 1986; Harter et al, 1995; reviewed by White et al, 1994). While non-genomic actions have been studied (see Introduction section 1.8), many of the genomic effects of calcitriol are mediated by the vitamin D receptor (VDR) and its interaction with the promoter region of target genes known as the vitamin D response element (VDRE), leading to direct transcriptional regulation by calcitriol, such as that observed for osteocalcin expression. The effects of this hormone in MG-63 osteoblast-like cells have been examined (Mahonen et al, 1990) and, as in osteoblastic cells, an induction of mRNA expression of osteocalcin and vitamin D receptor was seen following treatment of the cells with calcitriol.

In the studies described in this chapter, osteocalcin expression was used as an indicator of differentiation to the osteoblastic phenotype. It was also employed as a model in the study of vitamin D regulation of gene expression. As expected, calcitriol promoted osteocalcin expression. The increase in the expression of BMP-4, BMP-2, MSX-2 and osteocalcin following treatment of the cells with calcitriol is indicative of an overwhelming drive towards differentiation: even increased levels of BMP-2 and MSX-2 are insufficient

to prevent the expression of osteocalcin. Stimulation of osteocalcin mRNA expression was also seen following treatment with BMP-6, BMP-7 and TGF-beta. BMP-2 had a biphasic effect on osteocalcin expression. BMP-4 had no real effect on osteocalcin transcript levels and stimulated those of MSX-2. This strengthens the hypothesis that decreased BMP-2 levels allow differentiation to proceed by blocking an increase in MSX-2 levels and stimulating osteocalcin transcript expression, with higher levels of BMP-2 and BMP-4 preventing differentiation through the induction of the expression of MSX-2.

Expression of MSX-2 as an intermediate in the cascade of expression in the osteoblastic differentiation pathway was more extensively examined. As for BMP-4, induction of MSX-2 transcript expression by calcitriol was biphasic. Its stimulation by BMP-2 occurred with increasing dose. BMP-4 enhanced MSX-2 expression. This may have occurred indirectly, through the subsequent induction of BMP-2 by increased BMP-4 levels. The negative regulation of differentiation by MSX-2 (see Introduction section 1.6) is supported by the data in this chapter: induction of MSX-2 by BMP-2 or BMP-4 is coincident with a lack of stimulation in osteocalcin gene expression. However this inhibitory effect is not of sufficient magnitude to withstand the strong and direct stimulatory effect of calcitriol on osteocalcin mRNA expression. The finding that in adult human bone-derived cells, MSX-2 expression is consistently induced by both BMP-2 and BMP-4, concurs with observations in orofacial development, where BMP-2 and BMP-4 expression in the presumptive dental epithelium induces *Msx-1* and *Msx-2* expression in the mesenchyme (Thesleff et al, 1996), and in BMP-induced ectopic bone formation in Wistar rats where *Msx-2* expression was noted 6 days after implantation of BMP into the subcutaneous muscle layer (Iimura et al, 1994, b). BMP-6, BMP-7 and TGF-beta all enhanced MSX-2 expression maximally at lower doses in the ranges tested.

As previously outlined, studies into the effects of interactions between members of the TGF-beta superfamily have led to the idea that in vitro mixtures of BMPs have more potent effects upon the transcription of genes than single BMPs (Wang et al, 1990; Aono et al, 1995; Hazama et al, 1995; Lyons et al, 1995). Interactions between TGF-beta and BMP-7 blocked chondrogenesis in subconfluent primary rat calvarial cells (Asahina et al, 1993). BMP-2 was shown to affect the quantity and type of TGF-beta receptor expressed on the osteoblast cell surface, thus modulating the effects of TGF-beta (Centrella et al, 1995), and induced expression of TGF-beta in osteoblast-like cells (Zheng et al, 1994). Also, depending upon which was added first, calcitriol and TGF-beta abrogate the effects of the other agent in MG-63 cells (Iimura et al, 1994, a; Ingram et al, 1994). Therefore, it seemed appropriate to examine the effects of these agents in combination, compared to their individual effects, in MG-63 cells.

Investigations of the interactions between agents were performed using low doses of BMP-2, BMP-7, TGF-beta and calcitriol (tables 3.2a and 3.2b). The stimulation of

BMP-2 transcript expression by BMP-2 and its concurrent inhibition of the expression of BMP-4 transcripts confirms the observations made in previous experiments (table 3.1b) and supports the conclusion that an initial increase in BMP-2 mRNA expression further enhances levels of BMP-2 transcript, leading to a suppression of BMP-4 expression. In the presence of calcitriol, the stimulation of BMP-2 mRNA expression levels by BMP-2 is similar to those seen following treatment with calcitriol alone. This, and the observation that this combination stimulates osteocalcin mRNA expression to levels similar to those seen with calcitriol alone, again supports the conclusion that the expression of BMP-2 transcripts must be downregulated as differentiation proceeds.

BMP-7 and TGF-beta separately stimulated the levels of MSX-2 and osteocalcin transcript expression. It would seem appropriate in future work to extend these studies to include the effects of the agents studied on the expression of BMP-7 and TGF-beta, to clarify their position in the cascade.

Taken together, calcitriol in combination with members of the TGF-beta superfamily drives the cell towards a more differentiated phenotype, with increased mRNA levels of BMP-2, MSX-2 and osteocalcin. Interactions between members of the TGF-beta superfamily alone also promote differentiation, with expression of MSX-2 and osteocalcin transcripts ameliorated following treatment with any of the combinations tested. Effects on the transcription of the BMP-2 and -4 genes are more complex, and seem to indicate a more sedate progression through osteoblast-like differentiation.

To ascertain whether the effects of calcitriol on gene expression are osteoblast maturation-dependent, an investigation into the effects of calcitriol on gene expression in several osteoblast-like cell lines was performed. MC3T3-E1 cells are thought to model preosteoblasts, with MG-63 cells and ROS 17/2.8 cells modelling progressively more mature phenotypes.

When treated with calcitriol, MC3T3-E1 cells exhibited no change in *Msx-2* levels, suggesting a constitutive expression of this gene in these cells - this concurs with the proposal as outlined above that MSX-2 expression is seen in cells with a high proliferative potential and prior to the induction of osteoblastic differentiation. The reduction in MSX-2 mRNA levels seen in MG-63 cells treated with calcitriol, as consistently shown with Northern blot analysis, and the modest increase in osteocalcin expression indicates a progression towards a more mature phenotype. The modest increase in alkaline phosphatase levels, and the more pronounced increase in osteocalcin mRNA levels in ROS 17/2.8 cells treated with calcitriol are indicative of a more fully and terminally differentiated phenotype. The observation that *Msx-2* expression is increased by calcitriol treatment in ROS 17/2.8 cells confirms that seen by Hoffmann et al, 1994.

Following treatment with 10 nM calcitriol in MG-63 cells, Northern blot analysis consistently showed a decrease in MSX-2 mRNA levels, whereas in contrast, RT-PCR/Southern blotting consistently showed an increase in MSX-2 mRNA expression levels.

RNA from the same cells treated with and without calcitriol was analysed by Northern blot analysis and by RT-PCR, and decrease and increase with treatment was seen respectively. Reverse transcription reactions in the absence of reverse transcriptase were carried out in parallel to control for genomic contamination and any experiments showing contamination were excluded. Therefore, differences between Northern blot analysis and RT-PCR could not be attributed to genomic contamination. It is most likely that the RT-PCR is exquisitely sensitive: through the selection of the primer sets, this procedure could be detecting incomplete message in the process of being degraded or synthesized. The method of choice would be Northern blot analysis, as full length RNA only is detected using this procedure, or RNase protection assay, which requires smaller quantities of RNA.

Msx-2 is expressed in differentiating murine mesenchymal cells *in vitro* upon attainment of confluence (Nove et al, 1995): its expression is downregulated as development progresses. This, together with the observation that expression is observed in highly proliferative cells (Takahashi et al, 1996), suggests that MSX-2 is a gateway in osteoblast-like differentiation and, by its downregulation, may provide a switch from proliferation to committed osteoblastic differentiation. Therefore, understanding the mechanisms of its expression were examined.

Cycloheximide blocks *de novo* translation of mRNA into an amino acid sequence, thus abolishing the generation of fresh proteins in the cell. Actinomycin D abrogates the transcription of mRNA, thus subducing novel genetic expression. When introduced to MG-63 cells for 24 hours, the expected ablation of mRNA is seen following treatment with actinomycin D alone.

Treatment with calcitriol and actinomycin D together resulted in an abrogation of MSX-2 expression. This could suggest that one action of calcitriol on MSX-2 expression is independent of the generation of new transcripts. Treatment with cycloheximide alone stimulated MSX-2 expression, suggesting that the agent quashed the expression of a labile intermediate inhibitory protein. The modest increase in MSX-2 levels seen after introduction of cycloheximide and calcitriol in combination is suggestive of interference between the two agents, and perhaps indicates that the actions of both agents are within the same pathway whereby calcitriol could stimulate expression of the putative inhibitor to abrogate MSX-2 expression, and cycloheximide might override this to abolish expression of the inhibitor, thus releasing MSX-2 expression from calcitriol-induced downregulation.

The effects of inappropriate MSX-2 expression and of calcitriol treatment were examined in ROS 17/2.8 cells which were stably transfected by Jane Hodgkinson, UMDS, London, with a construct containing the MSX-2 coding sequence. As indicated, Msx-2 expression is modestly induced following calcitriol treatment of ROS 17/2.8 cells.



In these transformants, overexpression of MSX-2 was reduced in R2/11 cells, and doubled in R2/8 cells with calcitriol treatment. This observation is of great interest as the R2/8 cells appear to behave as the ROS 17/2.8 cells upon treatment with calcitriol, suggesting that incorporation of the MSX-2 sequence into the R2/8 genome did not occur in this transformant. This was confirmed by Jane Hodgkinson with a Southern of the R2/8 DNA (results not shown). She probed a Northern blot of R2/11 (untreated and calcitriol-treated) with an osteocalcin probe and found that osteocalcin levels were only modestly induced by calcitriol treatment when compared to untreated control (untreated=1, calcitriol treatment=1.4, results not shown). The R2/11 transformants, in downregulating their expression of MSX-2 and modestly increasing osteocalcin expression upon treatment with calcitriol treatment, appear to exhibit a pattern of gene expression similar to that of the MG-63 osteosarcoma cell line following treatment with calcitriol. Although further examination of a more detailed pattern of gene expression is required, including type I collagen, alkaline phosphatase, osteopontin, osteonectin and bone sialoprotein, this result raises the intriguing possibility that by overexpressing MSX-2, this transformant may have reverted to an earlier stage in the pathway of osteoblast-like differentiation.

The conclusions of these experiments investigating the effects of calcitriol and members of the TGF-beta superfamily are that there is a definite sequence of gene expression in the progression of the osteoblast-like cell through the differentiation pathway. Within the limitations of the techniques used, MSX-2 expression was seen to be regulated by BMP-2 and BMP-4 in adult human bone-derived cells, and may mediate the suppression of osteocalcin expression by these BMPs. Expression of MSX-2 appears to be a key switch in this differentiation cascade where, in the absence of a strong differentiation signal, expression of the gene must be downregulated in order for differentiation to proceed. This downregulation may be as a result of stimulation of the expression of an intermediate inhibitory protein. Calcitriol and members of the TGF-beta superfamily consistently stimulate the expression of osteoblast-related genes, the most potent combination for MG-63 cells being calcitriol plus BMP-7. This suggests that regulation of the expression of these genes is most likely to involve a series of interactions between the signalling molecules found in bone.

## **Chapter 4**

**Altered pattern of gene expression in adult human trabecular bone-derived cells following treatment with calcitriol and members of the TGF-beta superfamily**

## Introduction

As indicated in the introduction to the previous chapter, there are differences between osteoblast-like cell lines and normal osteoblastic cells: the sequence of gene expression in osteoblast-like cells lacks clearly defined stages in the progression from proliferation to terminal differentiation (Stein et al, 1990), whereas in osteoblasts, an inverse correlation between proliferation and differentiation is seen and there is stage-specific expression of osteoblast-related genes (reviewed by Aubin et al, 1993; Stein and Lian, 1993). The requirement for a structured extracellular matrix for development of the differentiated phenotype varies between osteoblast-like cell types, as does the regulation of gene expression by exogenous growth factors (Hauschka et al, 1988; Franceschi and Iyer, 1992).

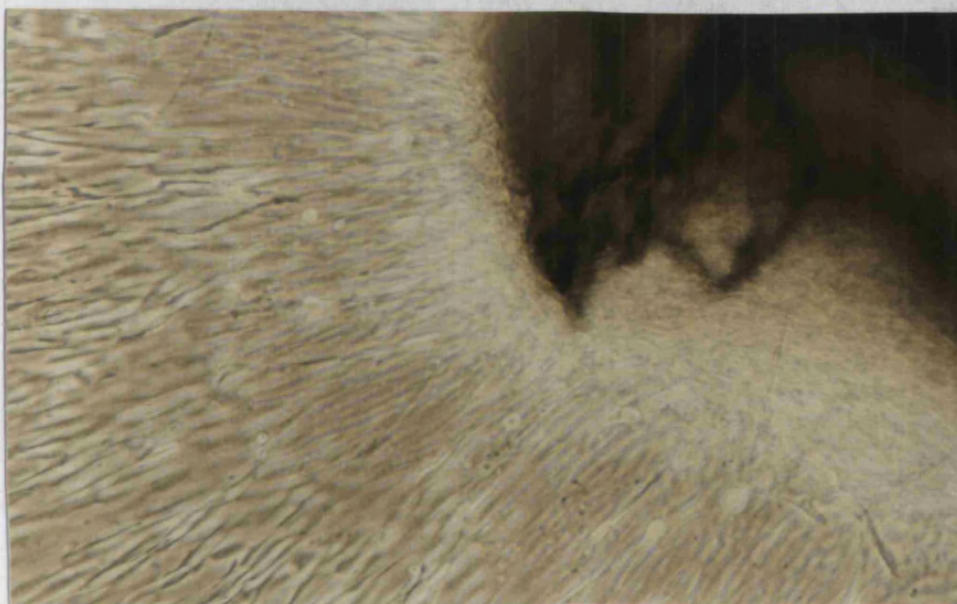
In order to compare gene expression in these osteoblast-like cells with that in differentiating osteoblasts, cells were cultured from explants of adult human trabecular bone under conditions previously described (see Materials and Methods, Beresford et al, 1986). In the following series of investigations, primary cultures were subcultured at a density of  $5 \times 10^3$ -  $10^4$  cells/  $\text{cm}^2$ .

### 4.1 Morphology of explant-derived cells in primary culture and at first passage

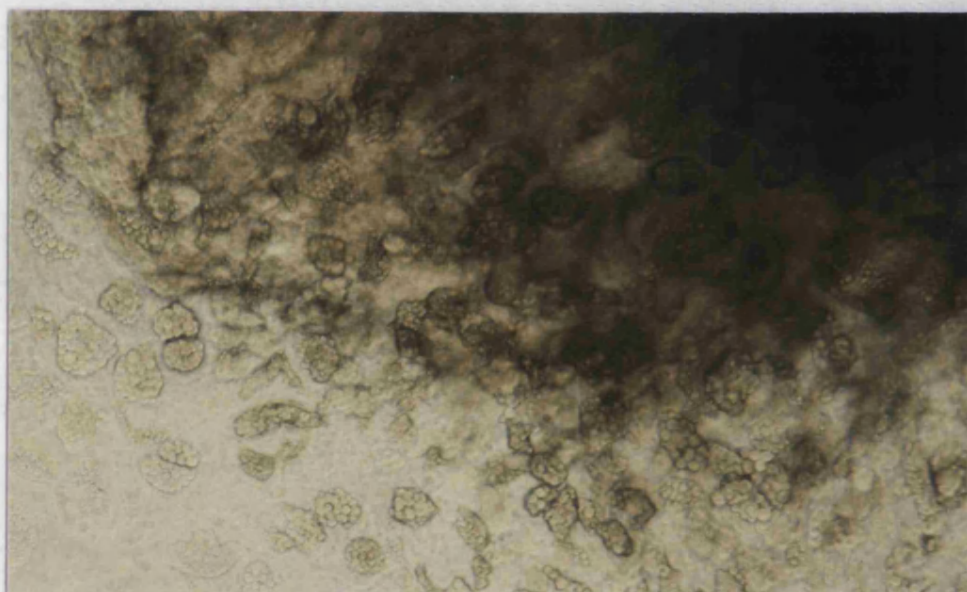
Figure 4.1 shows the outgrowth of osteoblastic cells from a trabecular bone chip in primary culture (photograph 4A). The cells migrate from the explant and proliferate to form a confluent cell layer. At this point, the cells are passaged to remove the bone chips as prolonged culture in the presence of the bone chips can result in the initiation of adipocyte differentiation (photograph 4B). The passaged cells are replated at a density of  $10^4$  cells/  $\text{cm}^2$  in a fresh flask and are treated at confluence with the agent(s) of choice prior to harvest of the cells and extraction of RNA. Photograph 4C shows cells approaching 50% confluence and photograph 4D shows cells at 100% confluence just before harvest of the cells.

### 4.2 Time course for the effects of calcitriol on the expression of osteoblast-related genes in cultures of human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum in the presence of 100  $\mu\text{M}$  ascorbate-2-phosphate, a stable analogue of ascorbic acid, were treated with 10 nM calcitriol as medium was replaced. RNA was extracted from cells at various timepoints thereafter up to 24 hours following treatment and analysed by RT-PCR for expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH mRNA transcripts (figure 4.2, top). The identities of the RT-PCR products were confirmed by Southern blotting using probes specific for the genes of interest (figure 4.2, bottom). In this experiment, volumetric analysis of the gels was carried out using densitometry to assess any changes in the



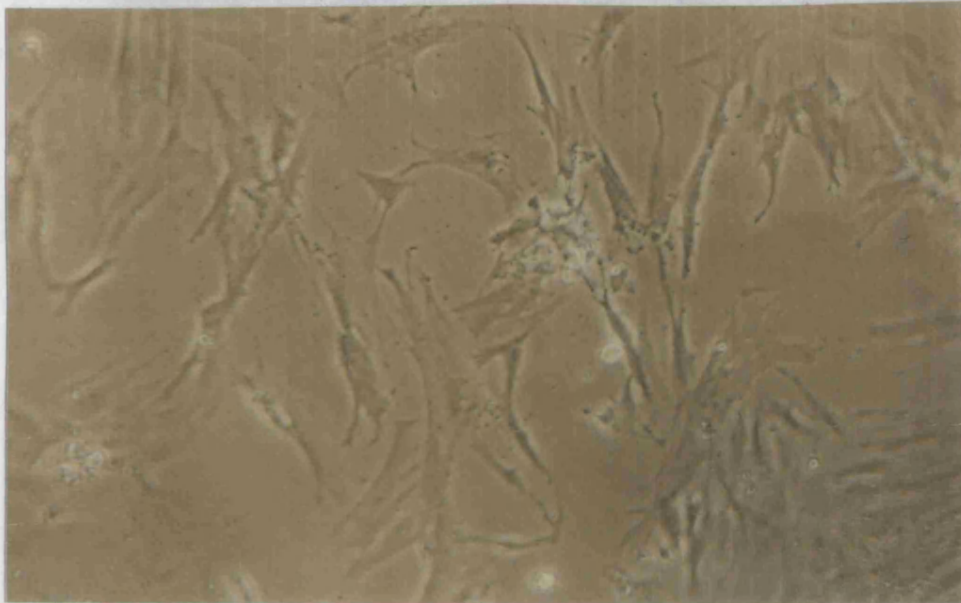
Photograph 4A: Outgrowth of cells from an adult trabecular bone-derived explant. These cells were maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate until confluent (after 4 weeks) at which point the cells were passaged and replated in the absence of the explants (original magnification x100).



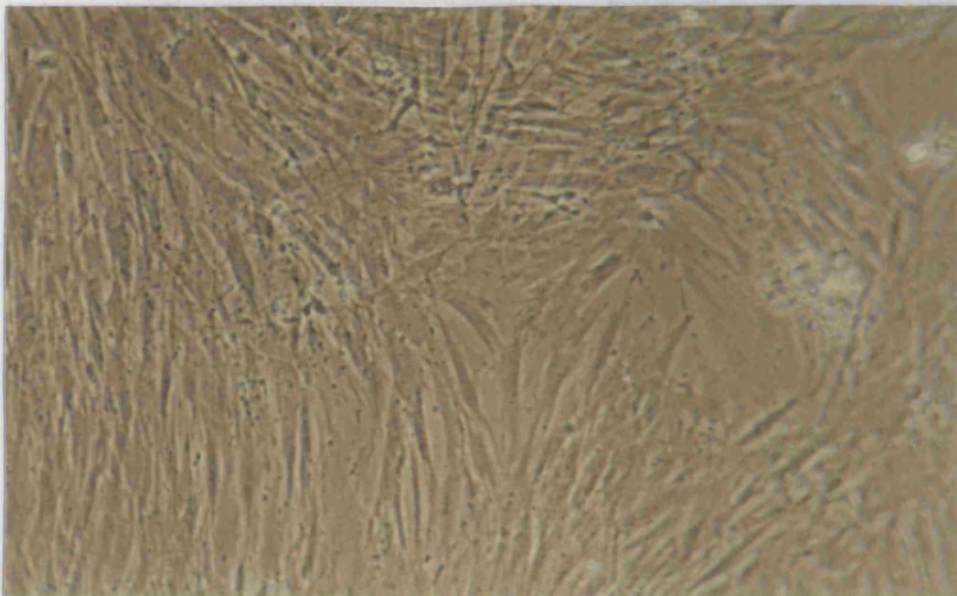
Photograph 4B: Outgrowth of adipocytic cells from an adult trabecular bone-derived explant. These cells were maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate and were cultured in the presence of the explant for 8 weeks (original magnification x100).

Figure 4.1a Morphology of explant-derived cells in primary culture





Photograph 4C: These cells were passaged and maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate. This photograph shows first passage cells at 50% confluence (original magnification x100).



Photograph 4D: These cells were passaged and maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate until almost confluent (after 4 weeks) at which point the cells were cultured in DMEM containing 2% fetal calf serum and ascorbate for 24 hours prior to treatment and harvest of RNA (original magnification x100).

Figure 4.1b Morphology of explant-derived cells at first passage

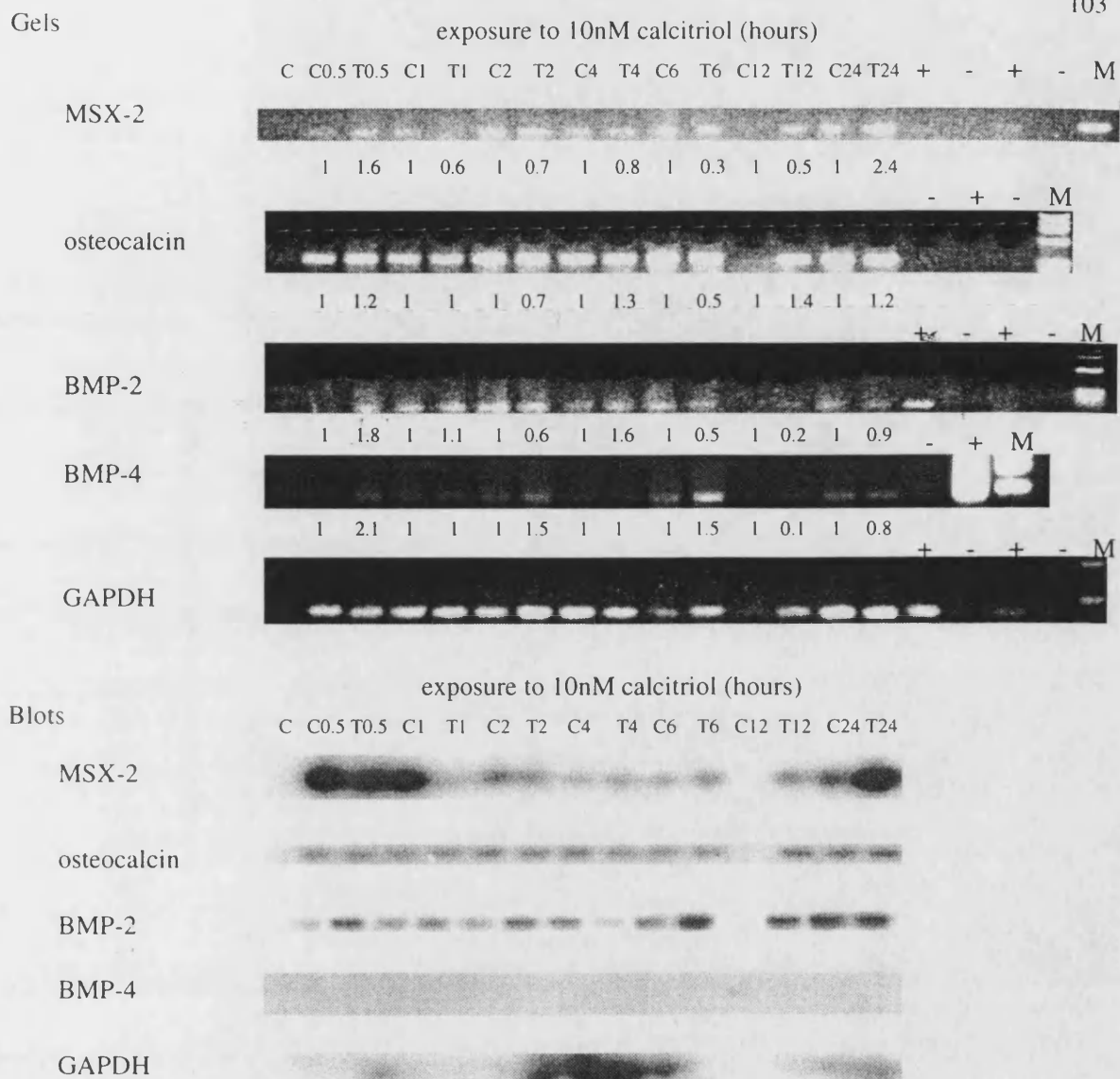


Figure 4.2 Time course for the effects of calcitriol on the expression of osteoblast-related genes in cultures of human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM containing 10% fetal calf serum in the presence of 100  $\mu$ M ascorbate-2-phosphate were cultured in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate supplemented with 10 nM calcitriol (T). RNA from these and from paired untreated control cells (C) was harvested at time intervals in the range 0-24 hours and subjected to RT-PCR for MSX-2 (35 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (40 cycles) and GAPDH (30 cycles) followed by Southern blotting. The gels were selected for analysis by densitometry as the blots for BMP-2 and GAPDH were inadequate for analysis. Densitometric values are seen in the above figure under the gels. This experiment was performed once.

	exposure to 10 nM calcitriol (hours)						
	0.5	1	2	4	6	12	24
mRNA							
MSX-2	1.6	0.6	0.7	0.8	0.3	0.5	2.4
osteocalcin	1.2	1.0	0.7	1.3	0.5	1.4	1.2
BMP-2	1.8	1.1	0.6	1.6	0.5	0.2	0.9
BMP-4	2.1	1.0	1.5	1.0	1.5	0.1	0.8

Table 4.1 Time course for the effects of calcitriol on the expression of osteoblast-related genes in cultures of human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate for 24 hours prior to treatment with 10 nM calcitriol. At a range of timepoints from 0.5- 24 hours, RNA was extracted and analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH. Densitometric analysis was normalised for GAPDH and the table above summarises the relative calcitriol-induced mRNA expression levels of all the genes tested when compared to untreated cells at the equivalent timepoint.

level of mRNA transcripts as the blot for GAPDH was of inadequate quality (see table 4.1).

Following addition of fresh medium, irrespective of the presence or absence of calcitriol, there was a rapid increase in the mRNA transcript levels of all genes examined: 30 minutes after replacement of the medium, treatment with calcitriol modestly stimulated expression of MSX-2 and osteocalcin and more strongly that of BMP-2 and BMP-4 mRNA transcripts when compared to that seen in untreated cells at the same timepoint. After 24 hours, calcitriol had increased expression of MSX-2 mRNA transcripts when compared to untreated control at the same timepoint.

Calcitriol is known to have pro-differentiation effects (Beresford et al, 1986). The downregulation of BMP-2 expression by addition of calcitriol therefore indicates that abrogation of the expression of this gene may be a necessary step in the calcitriol-induced differentiation of osteoblastic cells. Likewise, the enhancement of MSX-2 expression by calcitriol in this instance may be required in the initiation of osteoblastic differentiation.

As already reviewed in the Introduction (section 1.7), a vitamin D response element (VDRE) is present in the osteocalcin gene promoter. Transcription of the osteocalcin gene is therefore subject to direct regulation by calcitriol. However, after 24 hours' exposure to calcitriol, there was no noticeable effect on the production of osteocalcin mRNA transcripts when compared to that seen in untreated cells at the same timepoint. One possible explanation for this is that, amongst other growth factors and hormones, serum may contain calcitriol (Mahonen and Mäenpää, 1994), therefore mRNA expression of osteocalcin may already be maximally induced and addition of exogenous calcitriol may not further induce expression. This explanation may also hold true for BMP-4 mRNA expression. Therefore in all subsequent experiments, fresh medium was added at least 24 hours prior to the addition of exogenous calcitriol to allow for the subsidence of gene expression induced by the addition of serum. An additional step to minimise the effects of serum was to reduce the concentration of serum present in the replacement medium from 10% to 2% (Mahonen and Mäenpää, 1994).

#### 4.3 The effect of culture in the absence and presence of ascorbate on the response of human bone-derived cells to calcitriol

Ascorbate-2-phosphate (asp) is a stable analogue of ascorbic acid, which has been shown to be essential for the post-translational modification of collagen and therefore the production of a structured extracellular matrix (Franceschi and Iyer, 1992). In this experiment (figure 4.3), confluent first passage human trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and in the absence or presence of 100  $\mu$ M ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate as appropriate. The cells were



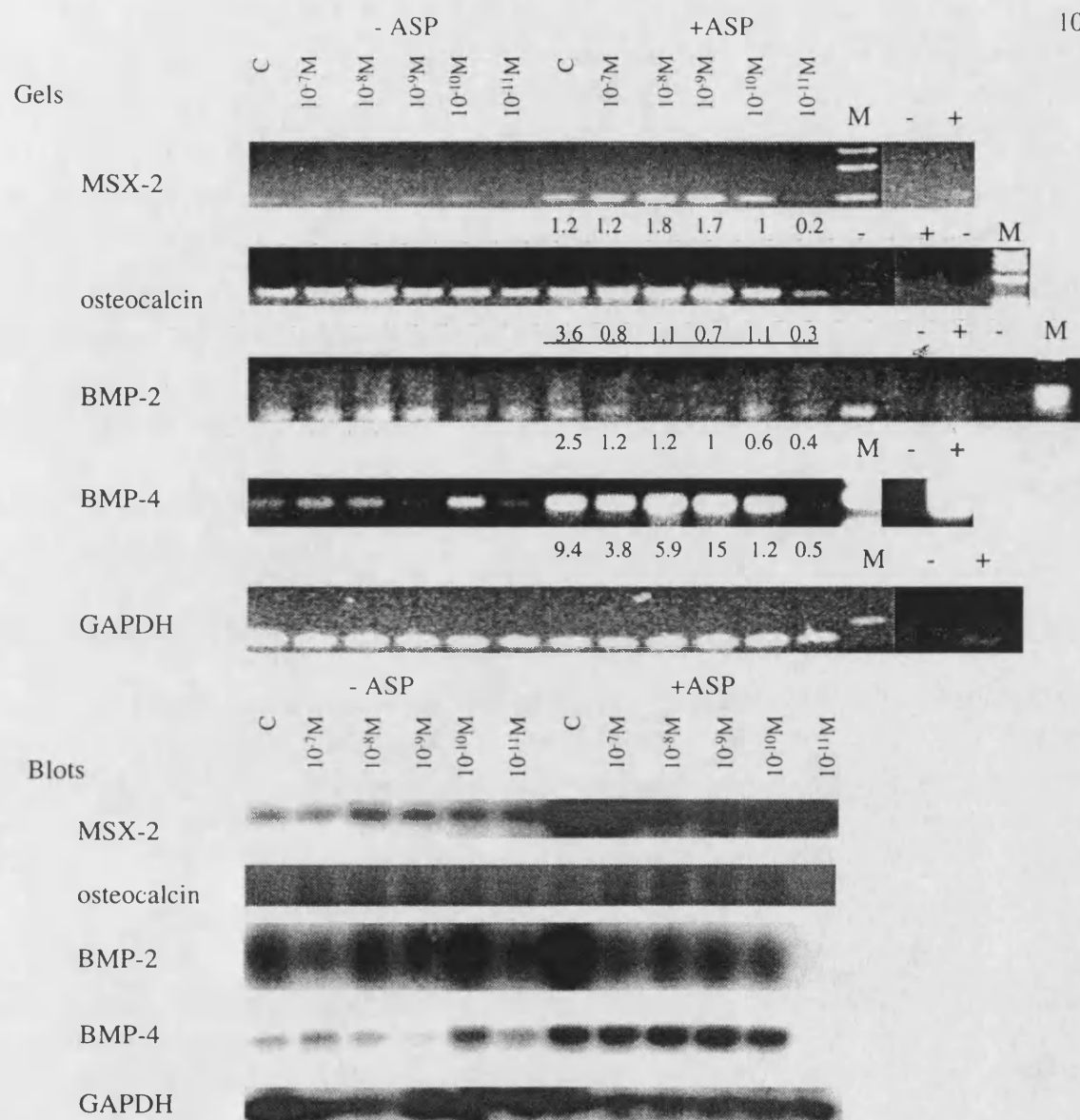


Figure 4.3 The effect of culture in the absence and presence of ascorbate on the response of human bone-derived cells to calcitriol

Confluent first passage trabecular bone-derived cells cultured continuously in the absence or presence of 100  $\mu$ M ascorbate-2-phosphate (-asp and +asp respectively) were fed with DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate 24 hours prior to treatment with increasing doses of calcitriol. After 24 hours' exposure to the agent, RNA was harvested and subjected to RT-PCR and Southern blotting to detect MSX-2 (35 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (35 cycles) and GAPDH (30 cycles) mRNA expression. Densitometry was then performed on the gels as the blots for MSX-2 and GAPDH were overexposed (values under each gel are derived from densitometric analysis and represent expression relative to cells treated without asp - for levels relative to untreated +/- asp, see tables 4.2a and b). The effects of ascorbate on calcitriol-induced osteocalcin expression were seen to be significant (underlined). This figure is representative of two independent experiments.

<u>Table A</u>	Final concentration of calcitriol (nM)					
	0	$10^{-7}$	$10^{-8}$	$10^{-9}$	$10^{-10}$	$10^{-11}$
MSX-2	1.2	1.2	1.8	1.7	1.0	0.2
osteocalcin	3.6	0.8	1.1	0.7	1.1	0.3
BMP-2	2.5	1.2	1.2	1.0	0.6	0.4
BMP-4	9.4	3.8	5.9	15	1.2	0.5

<u>Table B</u>	Final concentration of calcitriol (nM)					
	0	$10^{-7}$	$10^{-8}$	$10^{-9}$	$10^{-10}$	$10^{-11}$
MSX-2	1.0	1.3	0.9	0.7	0.8	0.7
osteocalcin	1.0	4.6	3	3.1	1.9	2.9
BMP-2	1.0	1.9	1.5	1.3	1.1	1.2
BMP-4	1.0	2.6	1.5	0.4	4.2	1.2

<u>Table C</u>	Final concentration of calcitriol (nM)					
	0	$10^{-7}$	$10^{-8}$	$10^{-9}$	$10^{-10}$	$10^{-11}$
MSX-2	1.0	1.2	1.1	1.2	0.8	0.1
osteocalcin	1.0	1.1	0.9	0.6	0.6	0.2
BMP-2	1.0	0.9	0.7	0.5	0.3	0.2
BMP-4	1.0	1.1	0.9	0.6	0.5	0.1

Table 4.2 The effect of culture in the absence and presence of ascorbate on the response of human bone-derived cells to calcitriol

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and cultured in the absence or presence of 100  $\mu$ M ascorbate-2-phosphate were treated with DMEM containing 2% fetal calf serum and ascorbate-2-phosphate as appropriate for 24 hours. The cells were then exposed to a range of doses of calcitriol for 24 hours. RNA was extracted and analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH mRNA expression. Densitometric analysis was performed on the gels and table A above summarises the relative mRNA expression levels of the genes of interest following culture in ascorbate-2-phosphate as compared to culture in its absence. For completeness, tables B and C record the relative expression levels of genes as compared to untreated control for cells cultured in the absence and presence of ascorbate-2-phosphate respectively.

then exposed to doses of calcitriol in the range  $10^{-7}\text{M}$  to  $10^{-11}\text{M}$  for 24 hours. RNA was extracted and analysed by RT-PCR and Southern blotting for the expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH. Densitometric analysis was performed on the gels and the results are shown in tables 4.2A- C.

In the presence of ascorbate-2-phosphate, and therefore of a functional extracellular matrix, the basal mRNA transcript levels of BMP-2, BMP-4 and osteocalcin are increased when compared to those seen in cells cultured in the absence of ascorbate (table 4.2A). MSX-2 transcripts are essentially unaffected in the ascorbate-treated cultures. This suggests that the extracellular matrix is necessary for terminal osteoblastic differentiation under untreated conditions.

Culture of cells in the presence of ascorbate causes the effects of calcitriol on the expression of mRNA transcripts to be biphasic: at higher doses, increase (MSX-2 and BMP-4) or no change (BMP-2 and osteocalcin) in mRNA transcript levels is seen when compared to cells cultured in the absence of ascorbate, and at the lower doses, downregulation of mRNA expression is seen for all genes tested.

For completeness, tables 4.2B and 4.2C show the effects of calcitriol on osteoblast-related gene expression in cells cultured in the absence or presence of ascorbate respectively when compared to cells not exposed to calcitriol. Following treatment with calcitriol, the increase in BMP-2, BMP-4 and osteocalcin mRNA transcript levels in the absence of ascorbate (table 4.2B) is in contrast to the inhibition of calcitriol-induced BMP-2, BMP-4 and osteocalcin mRNA expression seen in ascorbate-treated cultures (table 4.2C). The effects of calcitriol on the expression of these genes may be therefore dependent on the maturation stage of the cell.

Statistical analysis of these results can be seen in Appendix 3, null hypotheses 13 and 22.

#### 4.4 The effect of ascorbate on BMP-7-induced osteocalcin expression in human bone-derived cells

The previous experiment showed the necessity of an extracellular matrix for the regulation of gene expression by calcitriol and indicated a maturation-stage specific effect of calcitriol on the expression of BMP-2, BMP-4 and osteocalcin mRNA. In this investigation, the importance of the extracellular matrix was examined with regard to expression of osteocalcin following exposure to BMP-7 for 24 hours (figure 4.4). Confluent first passage human trabecular bone-derived cells, maintained in DMEM supplemented with 10% fetal calf serum and in the presence or absence of  $100\text{ }\mu\text{M}$  ascorbate-2-phosphate, were cultured for 24 hours in DMEM containing 2% fetal calf serum, and ascorbate if appropriate. The cells were then exposed to a range of doses of BMP-7 (5- 200 ng/ ml) for 24 hours prior to the extraction of RNA. This was analysed for the expression of osteocalcin and GAPDH mRNA transcripts by RT-PCR and Southern blotting.

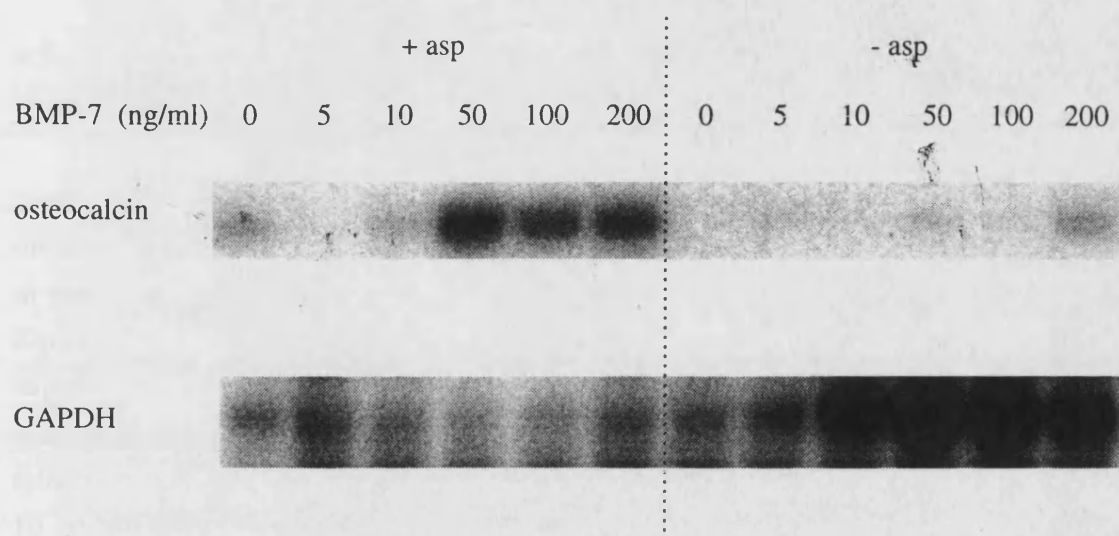


Figure 4.4 The effect of ascorbate on BMP-7-induced osteocalcin expression in human bone-derived cells

Confluent first passage trabecular bone-derived cells cultured constantly in DMEM containing 10% fetal calf serum and in the presence or absence of 100  $\mu$ M ascorbate-2-phosphate (+asp and -asp respectively) were fed with DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate 24 hours prior to treatment. They were then treated for 24 hours with a range of doses of BMP-7 (5-200 ng/ml) before RNA was harvested and RT-PCR and Southern blotting for osteocalcin and GAPDH was performed. This figure is representative of two independent experiments.

Basal levels of osteocalcin mRNA transcripts are increased in ascorbate-treated cultures as compared to those seen in untreated cultures. When differences in loading are taken into account, the ability of BMP-7 to induce osteocalcin mRNA expression appears to be absolutely dependent on the presence of ascorbate, and by inference, on the presence of a functional extracellular matrix. This indicates that induction of osteocalcin mRNA expression by BMP-7 is completely dependent on the maturation stage of the cell.

#### 4.5 The effect of cell density on the response of human bone-derived cells to treatment with calcitriol

As established in the previous experiments, the interaction between cell and matrix can be critical in determining the response of the cell to exogenous growth factors. It therefore seemed appropriate to investigate the importance of cell-cell contact in the expression of osteoblast-related genes in response to treatment with calcitriol. Equivalent numbers of passaged trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were plated at different densities and allowed to settle for 24 hours in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate before treatment for a further 24 hours with 10 nM calcitriol (see figure 4.5). RNA was analysed for the mRNA expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting. The blots were analysed by densitometry: the values for MSX-2 reflect differences in loading as bands were undetectable.

Osteocalcin steady state mRNA levels were increased at the higher cell density (table 4.3B), as was the magnitude of the increase observed in response to treatment with calcitriol. In contrast, basal expression of BMP-2 and BMP-4 mRNA was greater at the lower cell density: BMP-2 mRNA expression was decreased by calcitriol treatment whereas that of BMP-4 was increased. Following treatment of cells at the higher density with calcitriol however, BMP-2 and BMP-4 transcript expression was relatively increased when compared to levels in untreated cells plated at the same density, or with calcitriol-treated cells at the lower density (see table 4.3A).

Therefore, the response to calcitriol was seen to be cell density-dependent: mRNA transcript levels of BMP-2, BMP-4 and osteocalcin were stimulated following treatment with calcitriol at the higher cell density (see table 4.3A).

#### 4.6 The effects of bone morphogenetic proteins on osteoblast-related gene expression in human bone-derived cells

As seen in table 4.4, several experiments show that, in the presence of ascorbate, and therefore of a collagen-dependent extracellular matrix, calcitriol modestly increases the transcript expression of BMP-2 (average 1.7, range 0.5-3.5, table 4.4) and BMP-4 (average 1.8, range 0.8-3.1) and more strongly stimulates that of MSX-2 (average 2.2,

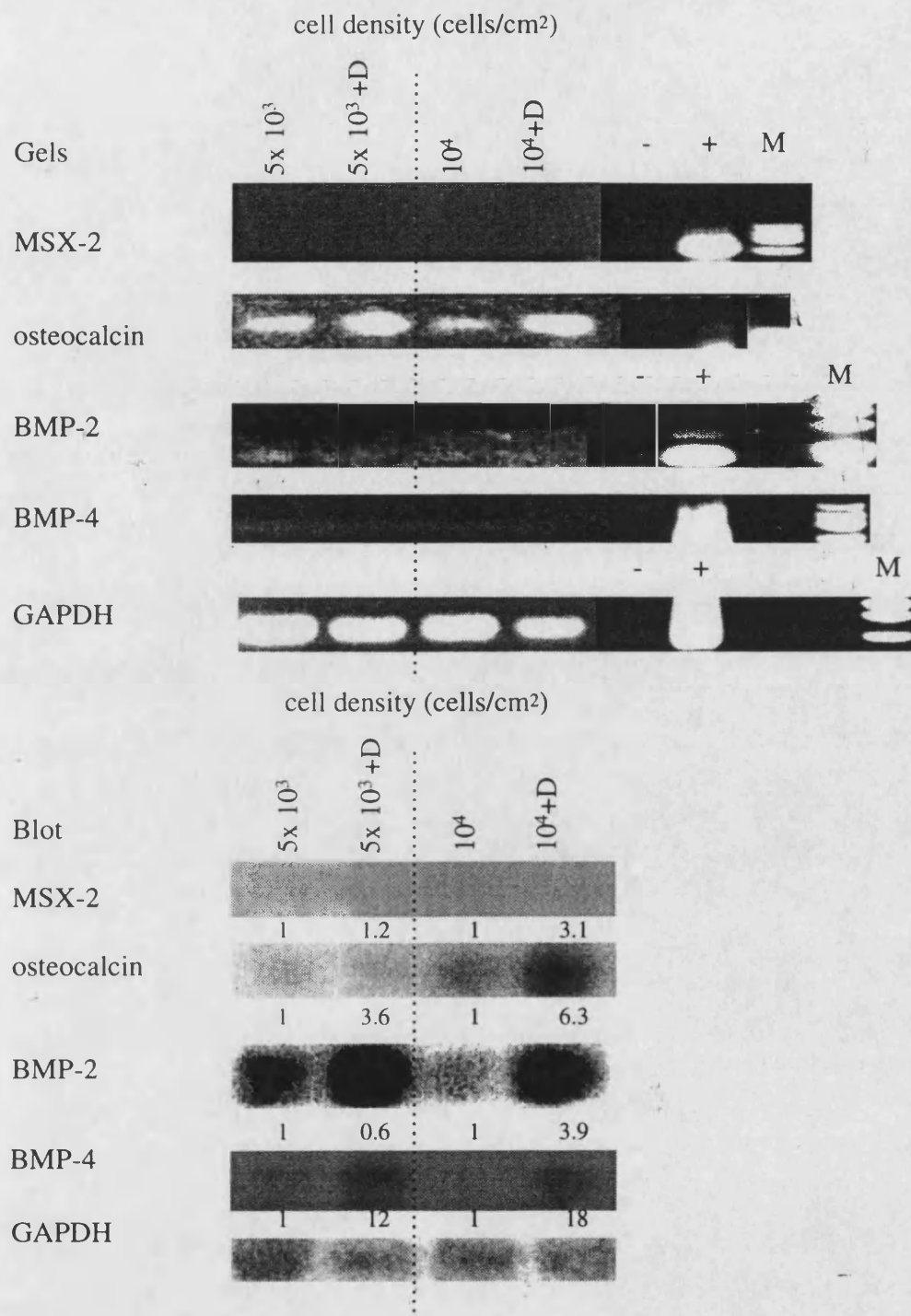


Figure 4.5 The effect of cell density on the response of human bone-derived cells to treatment with calcitriol

To investigate the influence of cell-cell communication in the regulation of gene expression, equivalent numbers of first passage trabecular bone-derived cells cultured constantly in DMEM with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were plated at the above densities and fed with DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate. They were allowed to settle for 24 hours and then were exposed to 10 nM calcitriol (D) for 24 hours. RNA was harvested and was analysed by RT-PCR and Southern blotting for MSX-2 (40 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (40 cycles) and GAPDH(30 cycles). This figure is representative of two independent experiments. Densitometric values relative to untreated cells at each density are

<u>Table A</u>		cell density (cells/ cm <sup>2</sup> )	
		5x 10 <sup>3</sup>	10 <sup>4</sup>
mRNA			
MSX-2		1.2	3.0
osteocalcin		3.6	6.3
BMP-2		0.6	3.9
BMP-4		12.3	17.9

<u>Table B</u>		cell density (cells/ cm <sup>2</sup> )	
		5x 10 <sup>3</sup>	10 <sup>4</sup>
mRNA	10 nM calcitriol		
	-	1.0	1.0
MSX-2	+	1.2	3.1
	-	1.0	2.5
osteocalcin	+	3.6	16.1
	-	1.0	0.4
BMP-2	+	0.6	1.4
	-	1.0	0.3
BMP-4	+	12.3	4.8

Table 4.3 The effect of cell density on the response of human bone-derived cells to treatment with calcitriol

First passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were plated at the above densities and allowed to settle for 24 hours before medium was replaced with DMEM containing 2% fetal calf serum and ascorbate. Cells were then treated with 10 nM calcitriol for a further 24 hours. RNA was extracted and analysed by RT-PCR and Southern blotting for expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH.

Table A summarises the relative calcitriol-induced expression levels of the genes of interest at the different cell densities as compared to untreated cells plated at the same density. For completeness, table B shows the relative expression levels of the genes of interest at the different cell densities and following treatment with or without calcitriol as compared to untreated cells plated at the lower density.

range 1-3.7, table 4.4) and osteocalcin (average 9.2, range 1-19). It seemed appropriate to examine the effects of bone morphogenetic proteins on the expression of these osteoblast-related genes to determine a sequence or cascade of gene expression in these cells. Confluent first passage human trabecular bone-derived cells, maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate, were plated at  $10^4$  cells/  $\text{cm}^2$  and cultured in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate for 24 hours prior to the addition of growth factors. The cells were then exposed to increasing doses of BMP-2 (5- 200 ng/ ml), BMP-4 (5- 150 ng/ ml), BMP-6 (10- 150 ng/ ml) and BMP-7 (10- 100 ng/ ml) for 24 hours. RNA was extracted and analysed for the expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH mRNA transcripts by RT-PCR (see figure 4.6a) and Southern blotting (see figure 4.6b). Densitometric analysis was performed upon the Southern blots. Where two values were obtained at the same dose, for example, untreated control and 20 ng/ ml BMP-2 (see figures 4.6a and 4.6b); an average of the two values is shown (table 4.5).

The effect of the bone morphogenetic proteins on MSX-2 transcript levels was clearly potent, with the greatest magnitude of increase in mRNA expression elicited by BMP-2, then BMP-4, BMP-6 and BMP-7. Osteocalcin mRNA levels were also increased following treatment with any of the BMPs tested, with BMP-2 treatment producing the greatest increase, followed by BMP-7, BMP-6 and BMP-4.

Clear, potent auto-regulation of BMP-2 transcript expression was observed. Expression of BMP-2 was increased following treatment with any of the BMPs tested, although to a lesser extent following treatment with BMP-6 and BMP-7.

Autoregulation was also observed for BMP-4, with greatest response at the lowest dose (5 ng/ ml) and a reduction of the magnitude of stimulation with increased dose (20- 150 ng/ ml) to steady state levels. BMP-2 treatment produced a very strong increase in levels of BMP-4 mRNA transcripts.

Statistical analysis of the results of these experiments can be seen in Appendix 3, null hypotheses 14 and 15.

#### 4.7 The effects of calcitriol and members of the TGF-beta superfamily, alone and when added in combination, on the expression of osteoblast-related genes in human trabecular bone-derived cells

The coincident expression of several BMPs in mouse and chick development suggests the possibility of interactive effects or of heterodimer formation between BMPs (Lyons et al, 1995; Barlow and Francis-West, 1997). Such combinations of BMPs or heterodimers may have more pronounced effects than their homodimers. Therefore, following on from the previous experiment where cells were exposed to individual BMPs, these experiments examine the effects on the expression of osteoblast-related genes of exposure to combinations of BMPs, the related agent transforming growth factor-beta-1 (TGF-beta) and calcitriol.



Factor	None	10 <sup>-8</sup> M calcitriol
mRNA		
MSX-2	1	2.2 (1-3.7)
osteocalcin	1	9.2 (1-19)
BMP-2	1	1.7 (0.5-3.5)
BMP-4	1	1.8 (0.8-3.1)

Table 4.4 The effects of calcitriol on osteoblast-related gene expression in human bone-derived cells

First passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate for 24 hours prior to the addition of 10 nM calcitriol. After 24 hours, RNA was extracted and analysed by RT-PCR and Southern blot for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH mRNA expression. Densitometric analysis was performed on the gels or blots as appropriate. The table above summarises the results of five independent experiments and shows median values (range shown in parentheses).

Gels

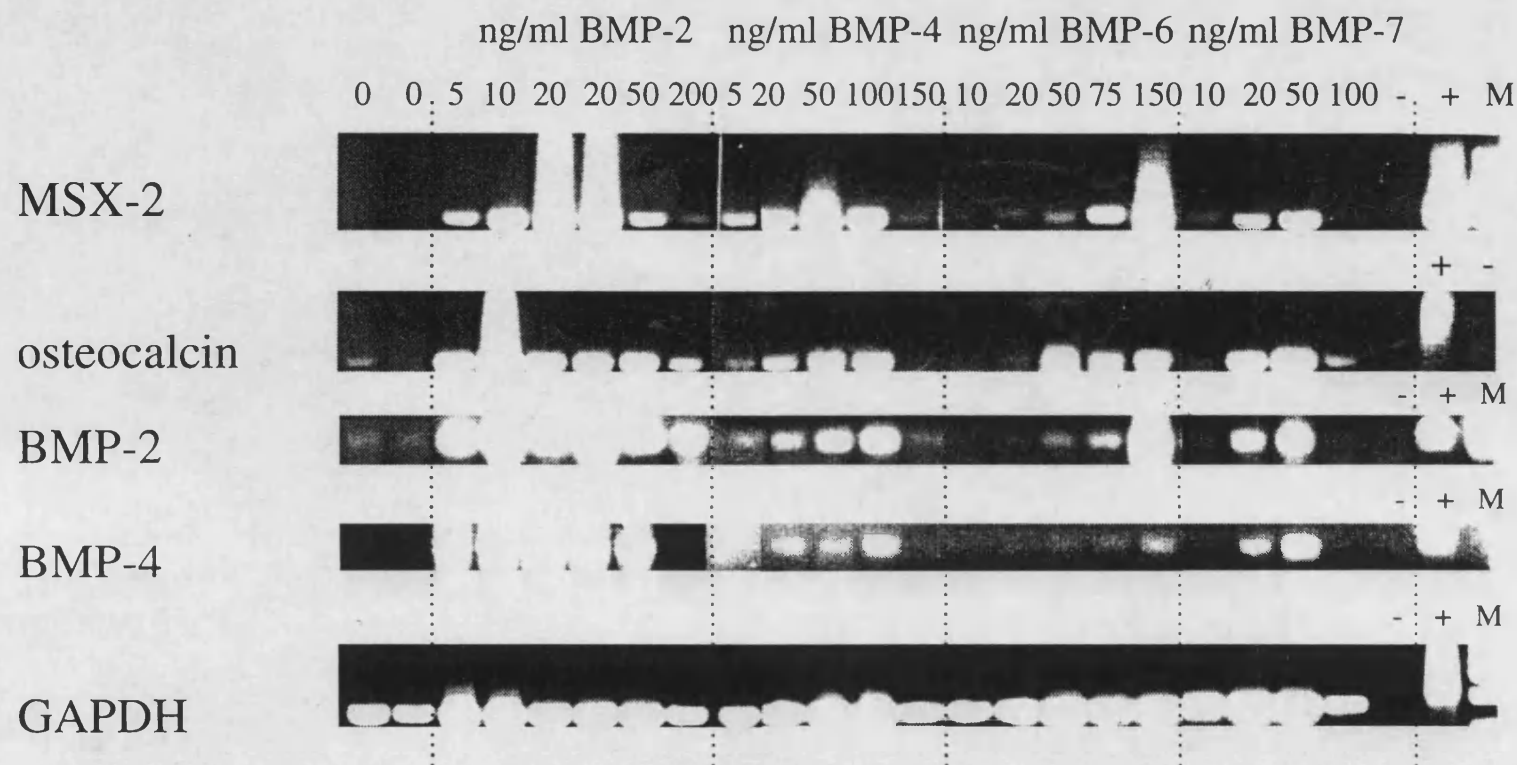


Figure 4.6a The effects of bone morphogenetic proteins on osteoblast-related gene expression in human bone-derived cells

Confluent first passage trabecular bone-derived cells cultured in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were fed with DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate 24 hours prior to treatment with the agents. Following 24 hours' exposure to the agents, RNA was harvested and analysed by RT-PCR for MSX-2 (35 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (35 cycles) and GAPDH (30 cycles), and Southern blotting. The figure above illustrates the ethidium bromide-stained gels and is representative of two independent experiments.

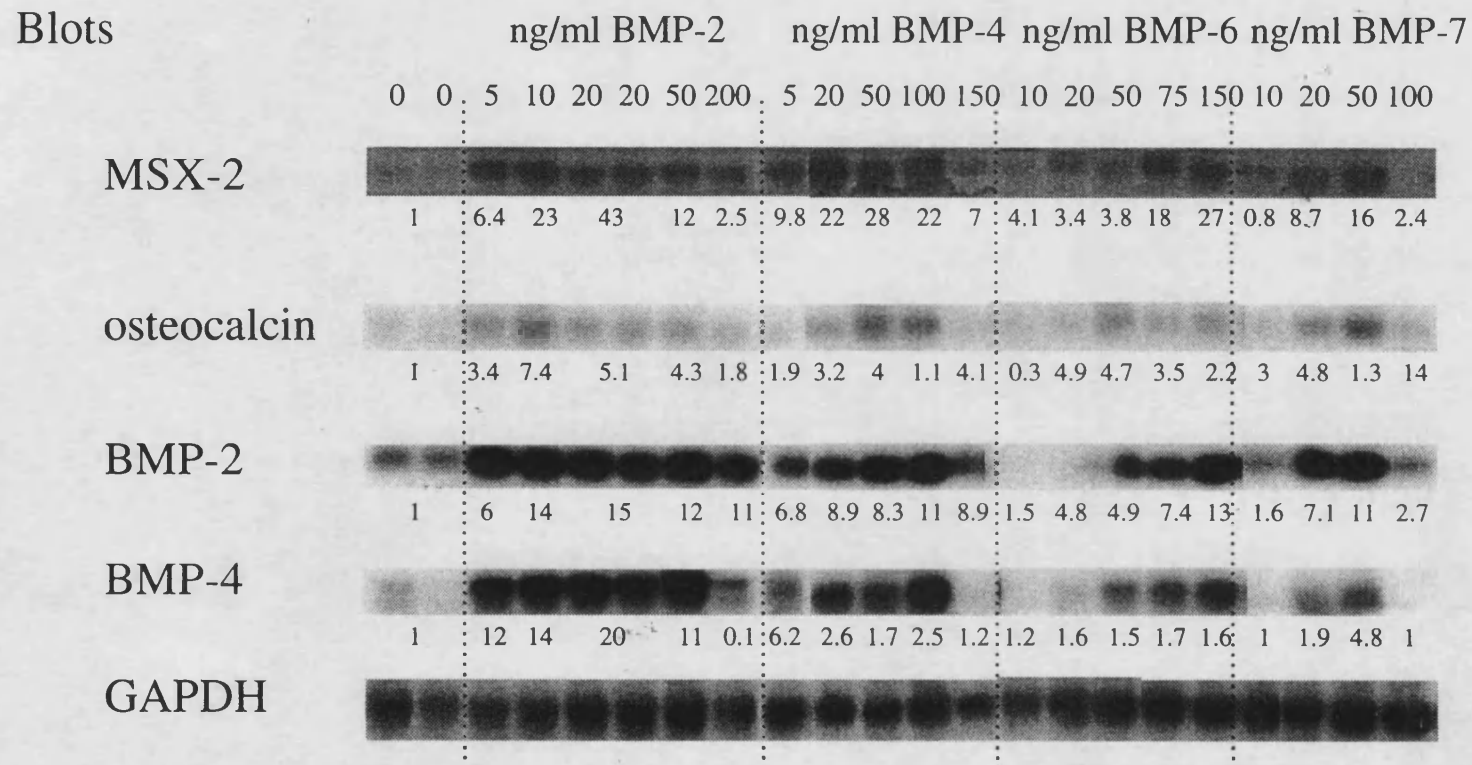


Figure 4.6b The effects of bone morphogenetic proteins on osteoblast-related gene expression in human bone-derived cells

This figure illustrates the Southern blots of the gels seen in figure 4.6a. Average densitometric values from two independent experiments are under each blot. Effects of BMP-2 on osteocalcin, BMP-2 and BMP-4 transcript expression, and of BMP-7 on that of osteocalcin and BMP-2 were seen to be significant.

		Dose (ng/ml)								
		0	5	10	20	50	75	100	150	200
mRNA	Factor									
MSX-2	BMP-2	1	6.4	23	43	12	nd	1.5 (0.8-2.2)~	nd	2.5
	BMP-4	1	9.8	nd	22	28	nd	22	7	nd
	BMP-6	1	nd	4.1	3.4	3.8	18	nd	27	nd
	BMP-7	1	nd	0.8	8.7	16	nd	2.4 (0.5-5.3)*	nd	nd
osteocalcin	BMP-2	1	3.4	7.4	5.1	4.3	nd	14 (12.8-15)~	nd	1.8
	BMP-4	1	1.9	nd	3.2	4	nd	1.1	4.1	nd
	BMP-6	1	nd	0.3	4.9	4.7	3.5	nd	2.2	nd
	BMP-7	1	nd	3	4.8	1.3	nd	14 (4.1-21)*	nd	nd
BMP-2	BMP-2	1	6	14	15	12	nd	4.0 (3.5-4.6)~	nd	11
	BMP-4	1	6.8	nd	8.9	8.3	nd	11	8.9	nd
	BMP-6	1	nd	7	4.8	4.9	7.4	nd	13	nd
	BMP-7	1	nd	1.6	7.1	11	nd	2.7 (1.6-4.1)*	nd	nd
BMP-4	BMP-2	1	12	14	20	11	nd	7 (6.9-8)~	nd	0.1
	BMP-4	1	6.2	nd	2.6	1.7	nd	2.5	1.2	nd
	BMP-6	1	nd	1.2	1.6	1.5	1.7	nd	1.6	nd
	BMP-7	1	nd	1	1.9	4.8	nd	1 (0.4-2)*	nd	nd

Table 4.5 The effects of bone morphogenetic proteins on the expression of osteoblast-related genes in human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate prior to the exposure for 24 hours to bone morphogenetic proteins. RNA was analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH transcripts. Densitometric analysis was performed to gauge changes in the levels of mRNA transcripts following normalisation using GAPDH levels. ~Average of two values obtained from independent experiments with the range of values shown in parentheses. \*Average of three values obtained from independent experiments with the range of values shown in parentheses. nd, not done.

Confluent first passage trabecular bone-derived cells, maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate, were cultured in DMEM containing 2% fetal calf serum and ascorbate for 24 hours prior to the addition of growth factors and agents. The cells were exposed to the agents, alone and in combination, for 24 hours. The doses selected were: 10 nM calcitriol, 100 ng/ml BMP-2/BMP-7 and 50pg/ml TGF-beta. RNA was then extracted and analysed for expression of mRNA transcripts of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting (figure 4.7). Densitometric analysis of the blots for MSX-2, BMP-2 and BMP-4 was performed, but gels were used for osteocalcin as the blots were over-exposed (see tables 4.6a and 4.6b).

When compared to untreated control, calcitriol alone stimulated expression of MSX-2 and osteocalcin transcripts, but did not affect the mRNA expression levels of the other genes. BMP-2 upregulated its own expression and that of BMP-4. BMP-7 stimulated BMP-2, MSX-2 and osteocalcin levels and had no effect on the mRNA expression of BMP-4. Treatment with TGF-beta alone elevated expression of all four genes.

Treatment with TGF-beta superfamily members in combination with calcitriol produced markedly different results (table 4.6a). Effects of BMP-2 in combination with calcitriol on the expression of BMP-2 and BMP-4 mRNA were similar to those seen following treatment with BMP-2 alone, whereas in contrast, BMP-2 in combination with calcitriol elicited an increase in MSX-2 mRNA expression and had a supra-additive effect on osteocalcin transcript levels when compared to the effects of either agent alone. BMP-7 in combination with calcitriol decreased levels of MSX-2 and BMP-4 transcripts, increased those of BMP-2 and had a supra-additive effect upon those of osteocalcin when compared with the action of either agent alone. Combining TGF-beta and calcitriol had no effect on BMP-2 transcript levels, and decreased those of BMP-4, when compared with TGF-beta alone. This combination produced a modest increase in MSX-2 and a supra-additive increase in osteocalcin mRNA expression levels when compared with the action of either agent alone.

The effects of members of the TGF-beta superfamily in combination were also examined (table 4.6b). BMP-2 and BMP-7 in combination decreased mRNA expression of BMP-2 and MSX-2 to levels below those seen following treatment with either agent alone. BMP-7 had an antagonistic effect on the stimulation by BMP-2 of BMP-4 transcript expression. In contrast, BMP-2 and BMP-7 stimulated osteocalcin transcript expression to levels similar to those seen following treatment with BMP-7 alone. BMP-2 and TGF-beta in combination reduced the expression of BMP-2 and BMP-4 to levels lower than those seen following treatment with either agent alone. Addition of TGF-beta and BMP-2 had no effect on the stimulation by TGF-beta alone on osteocalcin transcript expression. This combination stimulated MSX-2 mRNA expression to levels greater than those seen following treatment with either agent alone.

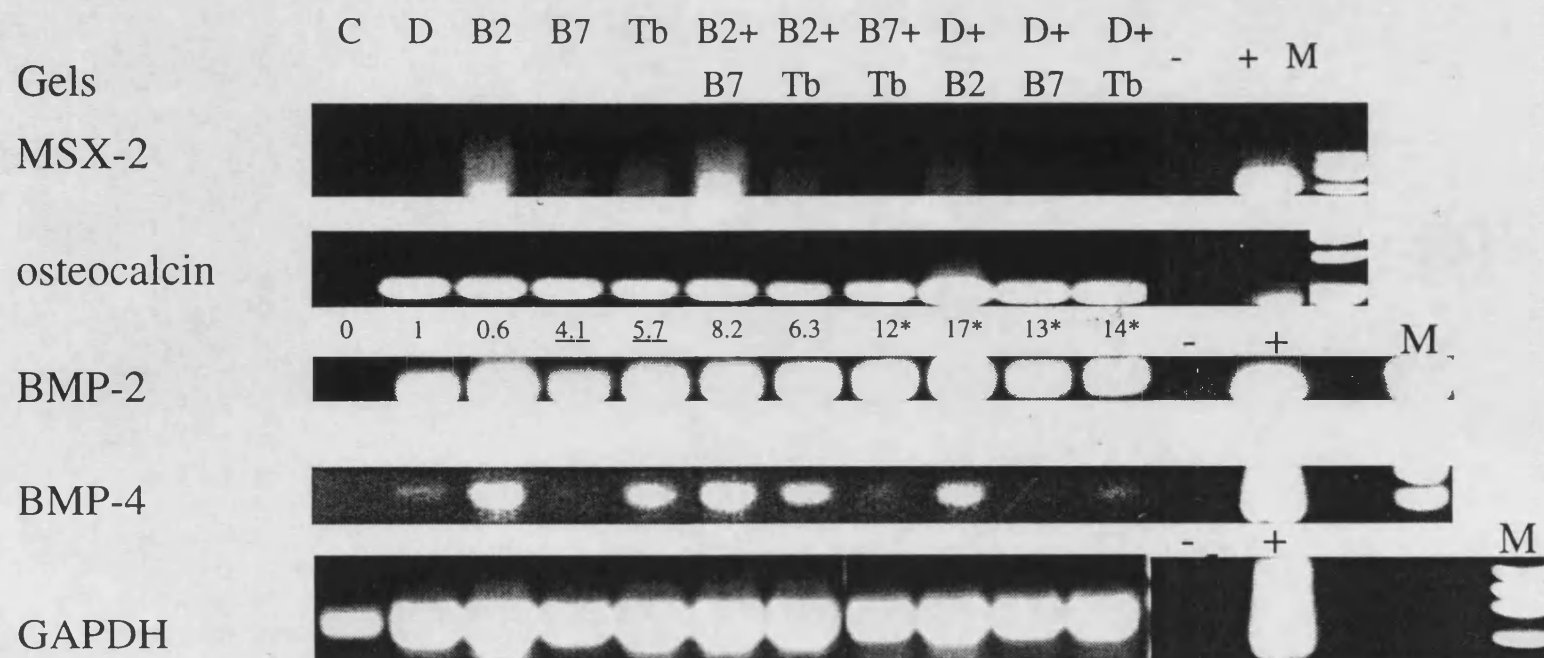


Figure 4.7a The effects of calcitriol and members of the TGF-beta superfamily, alone and when added in combination, on the expression of osteoblast-related genes in human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate for 24 hours prior to treatment with the following agents: untreated (C), 10 nM calcitriol (D), 100 ng/ml BMP-2 (B2) or BMP-7 (B7), and 50 pg/ml TGF-beta (Tb). Following exposure to these agents for 24 hours, RNA was harvested and analysed by RT-PCR for MSX-2 (35 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (35 cycles) and GAPDH (30 cycles) and Southern blotting. Densitometry was performed for osteocalcin (values below gel) as the blot was overexposed. Underlined values are significant in the first part of the Kruskal-Wallis test ( $H$ -values significant). \* Q values significant in two-part Kruskal-Wallis test. This figure is representative of two independent experiments.

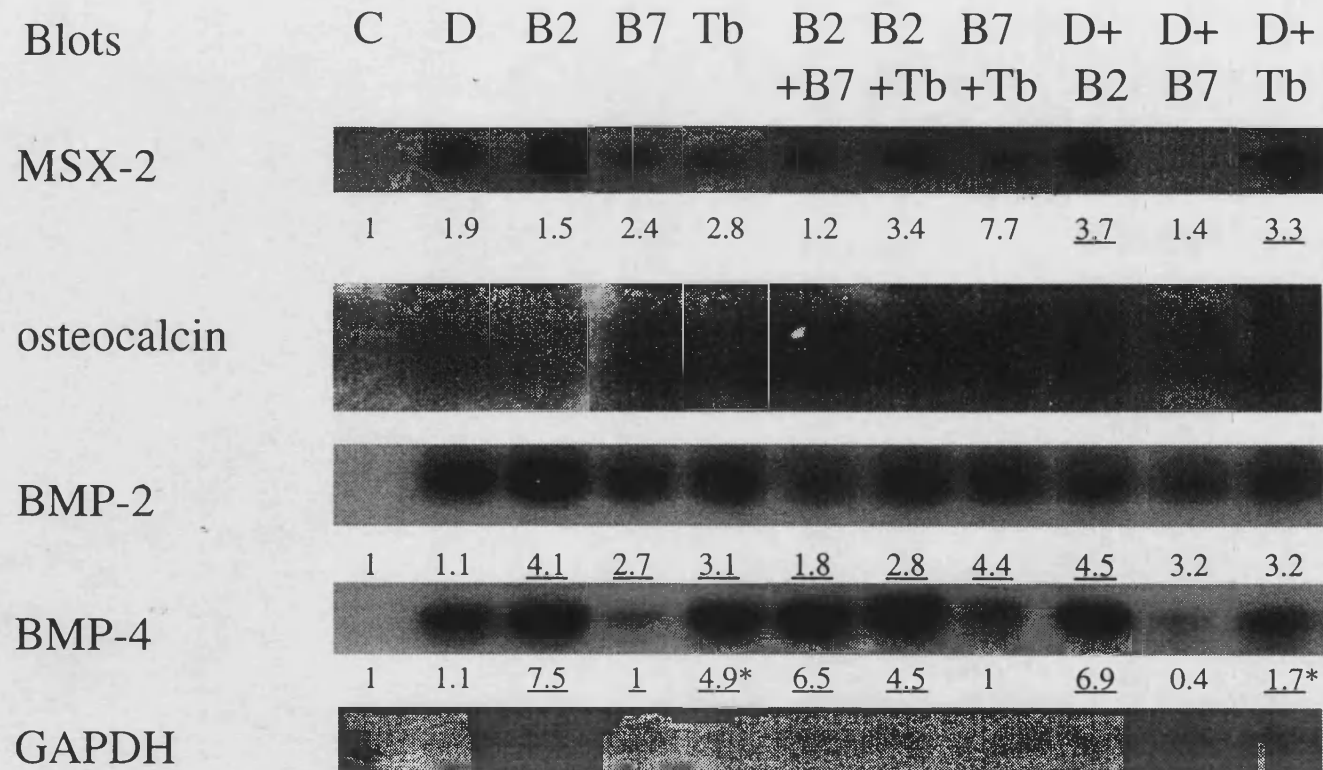


Figure 4.7b The effects of calcitriol and members of the TGF-beta superfamily, alone and when added in combination, on the expression of osteoblast-related genes in human bone-derived cells

This figure illustrates the Southern blots of the gels seen in figure 4.7a. Densitometry was performed on the blots for MSX-2, BMP-2 and BMP-4 (values below respective blots). Underline signifies that value is significant with the first part of the Kruskal-Wallis test (H value).

\* Q value shown to be significant by Kruskal-Wallis test. These are representative of two independent experiments.

Factor	None	10 nM calcitriol	10 nM calcitriol	BMP-2	BMP-7	TGF-beta
mRNA						
MSX-2	1	1.85 (1.8-1.9)~	-	1.5 (0.8-2.2)~	2.4 (0.5-5.3)*	2.8 (2.5-3.1)~
			+	3.7 (3.1-4.3)~	1.4 (0.9-1.8)~	3.3 (3-3.6)~
osteocalcin	0§	1 ~	-	0.6 (0.5-0.8)~	4.1 (1-16.5)*	5.7 (0.5-11)~
			+	17 (0.7-34)~	13 (0.4-26)~	14 (0.7-29)~
BMP-2	1	1.1 (0.9-1.2)~	-	4.1 (3.5-4.6)~	2.7 (1.6-4.1)*	3.1 (2.4-3.8)~
			+	4.5 (4.1-4.9)~	3.2 (2.8-3.6)~	3.2 (2.7-3.7)~
BMP-4	1	1.1 (0.8-1.4)~	-	7.5 (6.9-8)~	1 (0.7-2)*	4.9 (4.5-5.3)~
			+	6.9 (6.5-7.3)~	0.4 (0.1-0.7)~	1.7 (1.5-1.9)~

Table 4.6a The effects of calcitriol, alone or in combination with members of the TGF-beta superfamily, on the expression of osteoblast-related genes in adult human bone-derived cells

Confluent first passage trabecular bone derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100 µM ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. The cells were then exposed for 24 hours to the following concentrations of agents: 10 nM calcitriol, 100 ng/ ml BMP-2 or BMP-7, and 50 pg/ ml TGF-beta. RNA was analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH transcripts. The table above summarises the densitometric analyses: ~average of two values obtained from independent experiments with the range of values shown in parentheses; \*median of three values obtained from independent experiments with the range of values shown in parentheses. § indicates that the level of expression was below the limit of detection of the assay.



Factor	None	BMP-2	BMP-7	TGF-beta	BMP-2 +BMP-7	BMP-2 +TGF-beta	BMP-7 +TGF-beta
mRNA							
MSX-2	1	1.5 (0.8-2.2)~	2.4 (0.5-5.3)*	2.8 (2.5-3.1)~	1.2 (1.1-1.2)~	3.4 (1.4-5.3)~	7.7 (5.9-9.5)~
osteocalcin	0§	0.6 (0.5-0.8)~	4.1 (1-16.5)*	5.7 (0.5-11)~	8.2 (0.5-16)~	6.3 (0.6-12)~	12 (2-21)~
BMP-2	1	4.1 (3.5-4.6)~	2.7 (1.6-4.1)*	3.1 (2.4-3.8)~	1.8 (1.6-2)~	2.8 (2.7-3)~	4.4 (4.1-4.7)~
BMP-4	1	7.5 (6.9-8)~	1 (0.7-2)*	4.9 (4.5-5.3)~	6.5 (5.9-7.1)~	4.5 (4.1-4.9)~	1 (0.5-1.6)~

Table 4.6b The effects of members of the TGF-beta superfamily, alone or in combination, on the expression of osteoblast-related genes in adult human bone-derived cells

Confluent first passage trabecular bone derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. The cells were then exposed for 24 hours to the following concentrations of agents: 100 ng/ ml BMP-2 or BMP-7 and 50 pg/ ml TGF-beta. RNA was analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH transcripts. The table above summarises the densitometric analyses: ~average of two values obtained from independent experiments with the range of values shown in parentheses; \* average of three values obtained from independent experiments with the range of values shown in parentheses; § indicates that the level of expression was below the detection limit of the assay.

BMP-7 and TGF-beta in combination stimulated mRNA expression of BMP-2 and osteocalcin when compared with the action of either agent alone. This combination had a supra-additive effect on the expression levels of MSX-2. In contrast, BMP-4 transcript expression was unaffected by this combination: BMP-7 antagonised the stimulatory effects of TGF-beta on the mRNA expression of this gene.

Statistical analysis of these results can be seen in Appendix 3, null hypotheses 16-18, and 20.1-20.3.

#### 4.8 The effects of combinations of calcitriol, fibroblastic growth factor-2 and members of the TGF-beta superfamily on the expression of osteoblast-related genes in human bone-derived cells

The importance of members of the fibroblastic growth factor family in bone formation has been emphasised by the wide range of osseous malformations, some of which are lethal (see section 1.3), and which result from mutations in their receptors. Normally, FGF-2 is found in chondrocytes in the proliferating and upper hypertrophic regions of the growth plate, with resting zone cells showing little or no staining for this factor, and in the zone of Ranvier, which regulates latitudinal bone growth (Jingushi et al, 1995). Stimulation of bone growth on the periosteal surfaces was seen after injection of basic FGF (FGF-2) into the subcutaneous tissues overlying the calvariae of normal mice (Dunstan et al, 1993).

In culture, the continuous presence of FGF-2 is known to stimulate proliferation and inhibit the expression of the differentiated osteoblastic phenotype (Canalis et al, 1988; Locklin et al, 1995), although the pro-proliferative effects of FGF-2 were shown to be reversible (Oliver et al, 1990). Also, FGF-2 has been shown to interact positively with TGF-beta in chondrocyte culture, suggesting that these factors act through different pathways (O'Keefe et al, 1994). Therefore it seemed appropriate to examine the interactions of FGF-2 with calcitriol and members of the TGF-beta superfamily on osteoblast-related gene expression in trabecular bone-derived cells.

Confluent first passage human trabecular bone-derived cells, maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate, were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. The cells were then exposed to the following doses of agents, alone or in combination, for 24 hours: 2.5 ng/ ml FGF-2, 10 nM calcitriol, 100 ng/ ml BMP-2/BMP-7 and 50 pg/ ml TGF-beta. RNA was extracted and analysed for the expression of the mRNA transcripts of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting (figure 4.8). For the effects of BMP-2, BMP-7 and TGF-beta alone, see figure 4.7. Densitometric analysis of the gels was performed (table 4.7).

When compared to untreated control, calcitriol enhanced mRNA transcript levels of MSX-2 only. Treatment with FGF-2 alone stimulated expression of BMP-2 and MSX-2 mRNA and modestly increased that of BMP-4. FGF-2 and calcitriol in

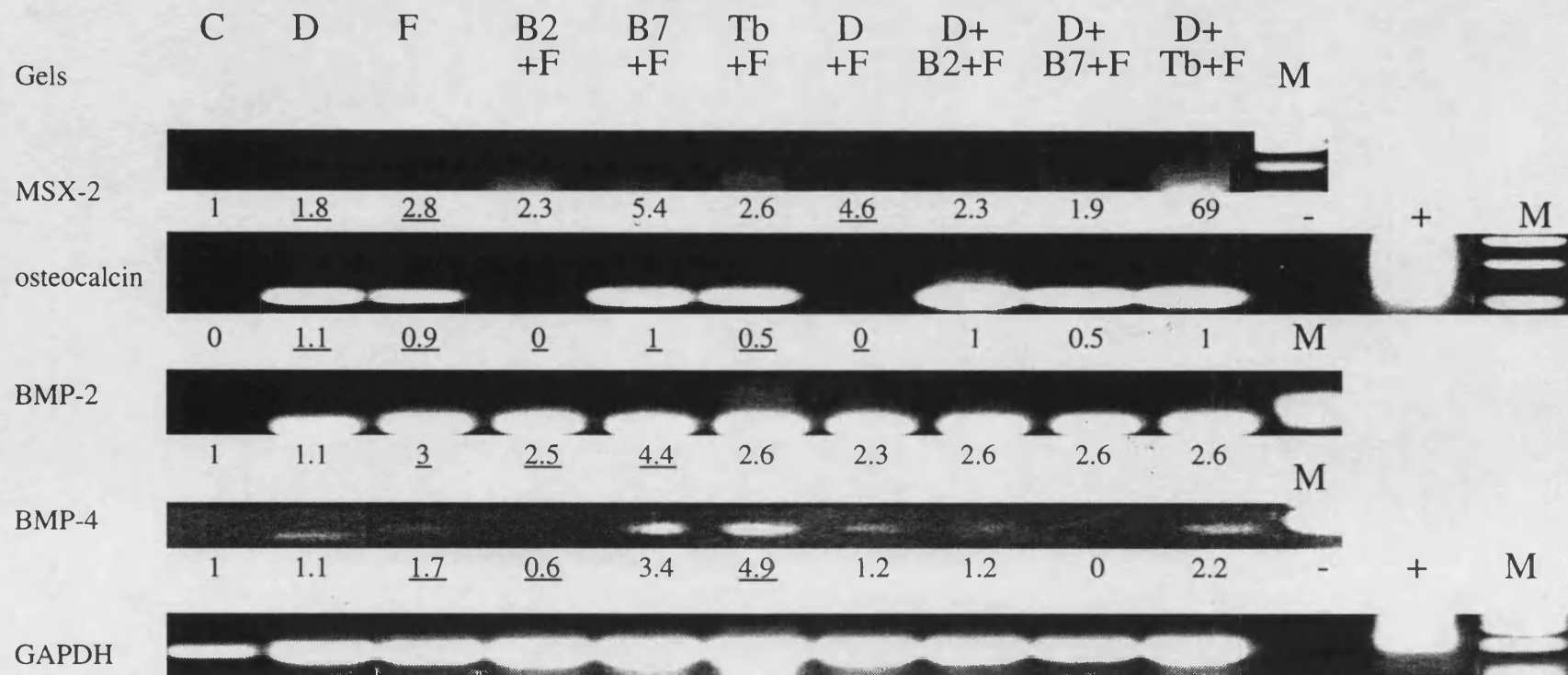


Figure 4.8a The effects of combinations of calcitriol, fibroblastic growth factor-2 and members of the TGF-beta superfamily on the expression of osteoblast-related genes in human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were incubated for 24 hours in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate. Cells were then exposed for 24 hours to agents, alone or in combination, at the following final concentrations: 10 nM calcitriol (D), 2.5 ng/ml fibroblastic growth factor-2 (F), 100 ng/ml BMP-2 (B2) or BMP-7 (B7), and 50 pg/ml TGF-beta (Tb). RNA was extracted, and RT-PCR for MSX-2 (35 cycles), osteocalcin (35 cycles), BMP-2 (35 cycles), BMP-4 (35 cycles) and GAPDH (30 cycles), and Southern blotting was performed. Densitometry was performed on the gels (values below gels). Underlined values indicate significant H values in the Kruskal-Wallis test. This experiment was carried out once.

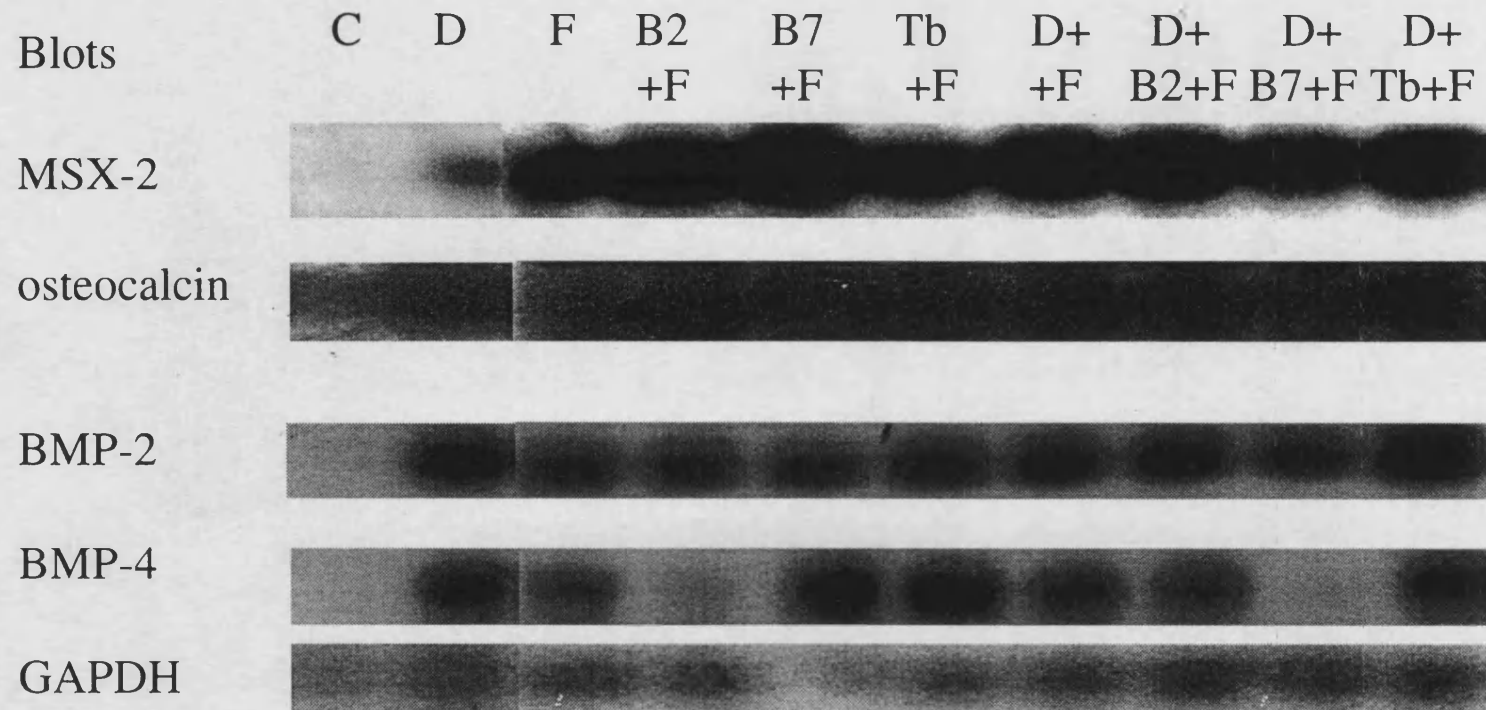


Figure 4.8b The effects of combinations of calcitriol, fibroblastic growth factor-2 and members of the TGF-beta superfamily on the expression of osteoblast-related genes in human bone-derived cells

This figure represents the Southern blots of the gels shown in figure 4.8a. This experiment was carried out once.

Factor	None	calcitriol	FGF-2	calcitriol+FGF-2			BMP-2	BMP-7	TGF-beta
mRNA					calcitriol	FGF-2			
					-	-	1.5 (0.8-2.2)~	2.4 (0.5-5.3)*	2.8 (2.5-3.1)~
					-	+	2.3	5.4	2.6
MSX-2	1	1.8 (1.8-1.9)~	2.8	4.6	+	-	3.7 (3.1-4.3)~	1.4 (0.9-1.8)~	3.3 (3-3.6)~
					+	+	2.3	1.9	69
					-	-	0.6 (0.5-0.8)~	4.1 (1-16.5)*	5.7 (0.5-11)~
					-	+	0	1	0.5
osteocalcin	0§	1.1 (1-1.2)~	0.9	0	+	-	17 (0.7-34)~	13 (0.4-26)~	14 (0.7-29)~
					+	+	1	0.5	1
					-	-	4.1 (3.5-4.6)~	2.7 (1.6-4.1)*	3.1 (2.4-3.8)~
					-	+	2.5	4.4	2.6
BMP-2	1	1.1 (0.9-1.2)~	3	2.3	+	-	4.5 (4.1-4.9)~	3.2 (2.8-3.6)~	3.2 (2.7-3.7)~
					+	+	2.6	2.6	2.6
					-	-	7.5 (6.9-8)~	1 (0.7-2)*	4.9 (4.5-5.3)~
					-	+	0.6	3.4	4.9
BMP-4	1	1.1 (0.8-1.4)~	1.7	1.2	+	-	6.9 (6.5-7.3)~	0.4 (0.1-0.7)~	1.7 (1.5-1.9)~
					+	+	1.2	0	2.2

Table 4.7 The effects of FGF-2, alone or in combination with calcitriol and/ or members of the TGF-beta superfamily, on the expression of osteoblast-related genes in human bone-derived cells

Confluent first passage trabecular bone derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100 µM ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. The cells were then exposed for 24 hours to the following concentrations of agents: 2.5 ng/ ml FGF-2, 10 nM calcitriol, 100 ng/ ml BMP-2 or BMP-7 and 50 pg/ ml TGF-beta. RNA was analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH transcripts. The table above summarises the densitometric analysis. ~Average of two values obtained from independent experiments with the range of values in parentheses. \*Average of three values obtained from independent experiments with the range of values shown in parentheses.

§ Band undetectable with this technique.

combination decreased osteocalcin mRNA expression to levels below the detection limit of this assay. Calcitriol antagonised the stimulatory effects of FGF-2 on the expression of BMP-2 and BMP-4 transcripts. In contrast, this combination produced an additive increase in the levels of MSX-2 mRNA expression.

FGF-2 and BMP-2 in combination strongly decreased the expression of osteocalcin and BMP-4 transcripts, and more modestly decreased levels of BMP-2 mRNA expression, when compared with the action of either agent alone. BMP-2 antagonised the stimulatory effects of FGF-2 on MSX-2 mRNA expression. Addition of calcitriol to this combination had no effect on the expression of MSX-2 and BMP-2 transcripts, stimulated the expression of BMP-4 transcripts to levels similar to those seen with calcitriol alone, and resulted in osteocalcin transcript levels similar to those seen following treatment with FGF-2 or calcitriol alone.

BMP-7 and FGF-2 in combination produced an additive increase in the expression of MSX-2 transcripts, and also increased BMP-2 and BMP-4 mRNA expression to levels greater than those seen following treatment with either agent alone, but resulted in levels of osteocalcin transcript similar to those seen following treatment with FGF-2 alone. Addition of calcitriol to the combination ablated expression of BMP-4 transcripts and reduced those of MSX-2 to levels similar to those seen following treatment with calcitriol alone. Calcitriol antagonised the stimulatory effect of FGF-2 and BMP-7 in combination on the expression of BMP-2 transcripts and reduced still further the levels of osteocalcin transcript expression.

FGF-2 and TGF-beta in combination had no effect on MSX-2, BMP-2 and BMP-4 transcript levels when compared to the action of TGF-beta alone. This combination produced a reduction in osteocalcin mRNA levels when compared to the effect of either agent alone. Addition of calcitriol to the combination had a potent effect on the expression of MSX-2 mRNAs, with a dramatic increase in the levels of transcripts. Addition of calcitriol, FGF-2 and TGF-beta had no effect on the level of BMP-2 transcripts when compared to the action of FGF-2 and TGF-beta in combination, whereas BMP-4 mRNA levels were decreased. Following combined treatment, osteocalcin transcript levels were similar to those seen after treatment with calcitriol alone.

Statistical analysis of these results can be seen in Appendix 3, null hypotheses 19 and 21.

#### 4.9 Effects of cycloheximide and actinomycin D treatment on calcitriol-regulated osteoblast-related gene expression in human bone-derived cells

As stated earlier, and in the Introduction (section 1.7), the osteocalcin promoter contains a vitamin D response element (VDRE). This has been studied extensively and is considered as a model of vitamin D-regulated gene expression (reviewed by Pike et al, 1993). It was therefore considered to be appropriate to gain some understanding of

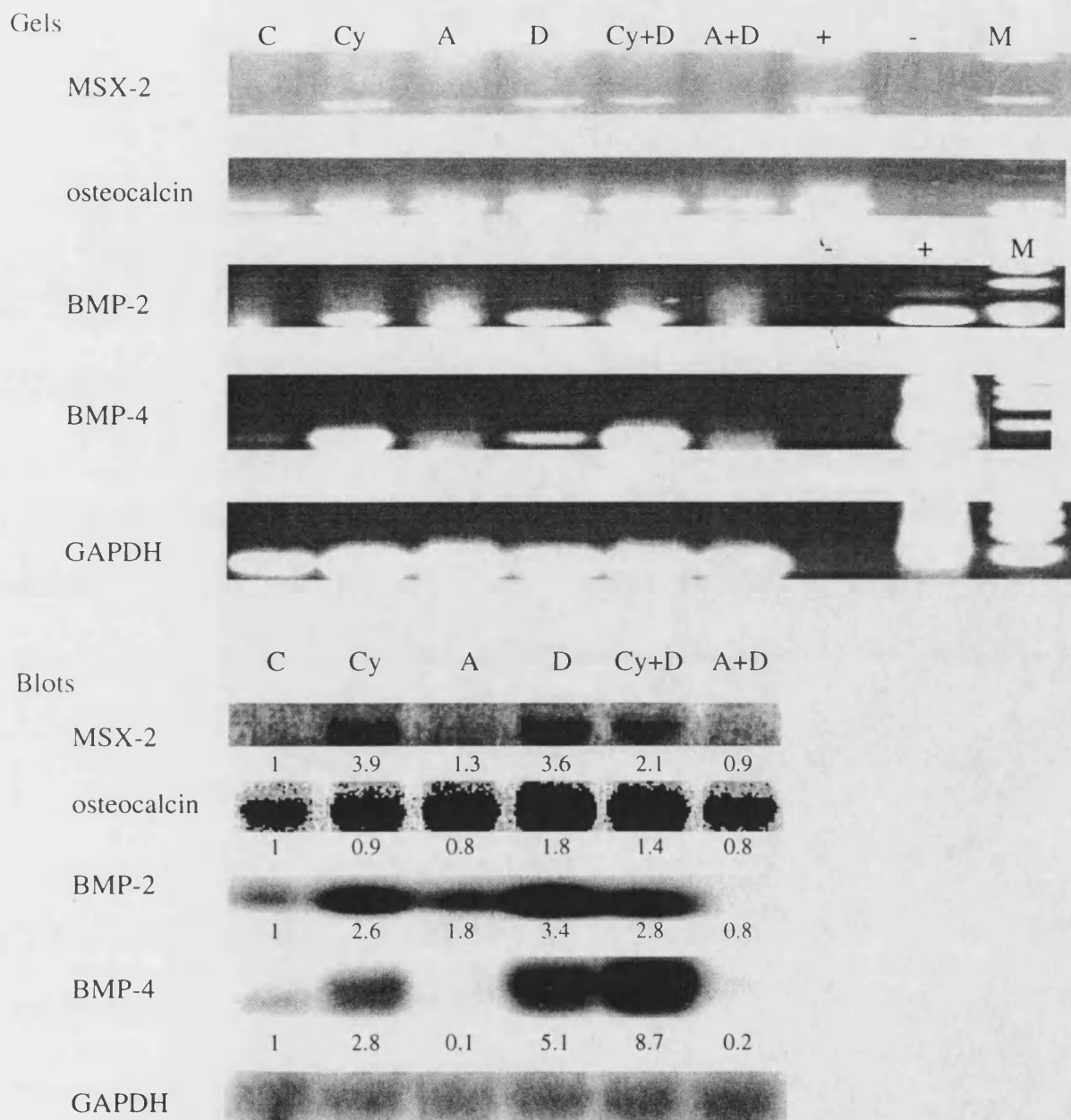


Figure 4.9 Effects of cycloheximide and actinomycin D treatment on calcitriol-regulated osteoblast-related gene expression in human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate for 24 hours prior to the addition of the agents in the following concentrations: untreated (C), 10 nM calcitriol (D), 2.5  $\mu$ g/ml cycloheximide (Cy) and 250 ng/ml actinomycin D (A). Following exposure to the agents for 24 hours, RNA was harvested and RT-PCR for MSX-2 (35 cycles), osteocalcin (30 cycles), BMP-2 (35 cycles), BMP-4 (35 cycles) and GAPDH (30 cycles), and Southern blotting was performed. Densitometry was performed on the blots (values below each blot). This experiment was carried out once.

Factor	none	2.5 mg/ ml cycloheximide	250 ng/ ml actinomycin D	10 nM calcitriol	2.5 mg/ ml cycloheximide + 10 nM calcitriol	250 ng/ ml actinomycin D + 10 nM calcitriol
mRNA						
MSX-2	1	3.9	1.3	3.6	2.1	0.9
osteocalcin	1	0.9	0.8	1.8	1.4	0.8
BMP-2	1	2.6	1.8	3.4	2.8	0.8
BMP-4	1	2.8	0.1	5.1	8.7	0.2

Table 4.8 The effects of calcitriol, cycloheximide and actinomycin D on the expression of osteoblast-related genes in human bone-derived cells

Confluent first passage trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate. The cells were then exposed to the agents for 24 hours. RNA was extracted and analysed by RT-PCR and Southern blotting for mRNA transcripts of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH. The table above represents the values obtained following densitometric analysis of the gels or blots from one experiment.



the regulation by calcitriol of the other genes of interest. Cycloheximide, an inhibitor of protein synthesis, and actinomycin D, an inhibitor of transcription and thereby of mRNA synthesis, were selected as agents for use in the examination of the direct or indirect regulation of expression of the genes of interest by calcitriol.

Confluent first passage trabecular bone-derived cells, maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate, were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. The cells were then exposed to the following concentrations of agents for 24 hours: 2.5  $\mu$ g/ml cycloheximide, 250 ng/ml actinomycin D and 10 nM calcitriol. RNA was harvested and analysed for the mRNA expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting (see figure 4.9). Densitometric analysis of the blots was performed (table 4.8).

Cycloheximide treatment enhanced expression of BMP-2, BMP-4 and MSX-2 transcripts and had no effect on levels of osteocalcin mRNA compared to untreated control. This suggests that in untreated cells, an inhibitory protein prevents the mRNA expression of BMP-2, BMP-4 and MSX-2, but that no such inhibitor affects osteocalcin expression. Actinomycin D treatment abrogated expression of BMP-4 and more modestly inhibited that of osteocalcin, suggesting that the half-lives of these transcripts is relatively short. When compared to untreated control, actinomycin D treatment fostered an increase in the transcript levels of BMP-2 and MSX-2, suggesting that the transcription of an inhibitor or of a degrading molecule was blocked with this treatment. Calcitriol stimulated mRNA expression levels of all four genes.

When compared to the effects of calcitriol alone, the combination of cycloheximide and calcitriol resulted in a smaller magnitude of increase in the expression of BMP-2 and MSX-2, suggesting some level of interference between the two agents. One possible explanation could be that the formation of the vitamin D receptor complex, or its interaction with the basal transcription apparatus, is affected by the introduction of cycloheximide, resulting in fewer transcripts than would be present following treatment with calcitriol alone. The production of other accessory molecules could also be affected. The effect of this combination on BMP-4 mRNA transcript levels was greater than that observed following treatment with cycloheximide or calcitriol alone, indicating that calcitriol induction of BMP-4 mRNA expression may be independent of the regulation by an intermediate labile protein, and that the BMP-4 promoter may contain a vitamin D response element (no evidence in literature). Cycloheximide and calcitriol together had a similar effect on osteocalcin mRNA levels to that seen following treatment with calcitriol alone, confirming that calcitriol-induced expression of osteocalcin mRNA is direct.

Treatment with actinomycin D and calcitriol in combination resulted in a pronounced decrease in the levels of mRNA transcripts for MSX-2 and BMP-2 when compared to the effects of either agent alone, suggesting that one function of calcitriol

may be to increase the turnover of these transcripts. Increased production and degradation of transcripts in this instance would be observed only as increased degradation due to the absence of de novo transcription, as blocked by actinomycin D. Osteocalcin and BMP-4 mRNA levels are unchanged following treatment with actinomycin D and calcitriol when compared to treatment with actinomycin D alone, suggesting that calcitriol does not affect the stability of these transcripts.

## Discussion

As stated previously, the limitations of the RT-PCR technique as employed in the course of this research are such that absolute quantitation is impossible. The emphasis of this discussion is therefore on the consistent changes in direction in the expression of transcripts of osteoblast-related genes observed using this technique, and not on changes in magnitude.

The sequence of gene expression in differentiating osteoblasts is more complex than that shown previously for osteoblast-like cells: factors such as cell density and interaction with the extracellular matrix appear to have a critical role in the regulation of osteoblastic gene expression.

As shown in this results chapter, cell density has a crucial part in the differentiation of trabecular bone-derived cells: compared with cells plated at the lower density, those plated at the higher density displayed a more differentiated phenotype, where BMP-2 expression was downregulated and osteocalcin expression was increased. Upon addition of calcitriol to the lower density cells, a similar pattern was observed. Treatment with calcitriol of cells at the higher density resulted in increased expression of these genes when compared to untreated control at the same density, to untreated control at the lower density or to treated cells at the lower density. This pattern is similar to that seen in MG-63 cells treated with high doses of calcitriol when compared to untreated cells and is suggestive of a strong drive towards differentiation. One possible explanation for this regulation is that cells will proliferate or differentiate according to intercellular communication. This may occur through an increase in the quantity of signalling molecules accumulating in the medium with increasing cell number, or more directly through gap junctional communication between neighbouring cells as the population grows more dense. Indeed, in cultured differentiating murine mesenchymal cells, the expression of *Msx-2* was seen to be dependent on the cell density as expression was induced only upon the cells reaching confluence (Nove et al, 1995). Therefore, as the trabecular bone-derived cells were harvested 48 hours after plating at  $5 \times 10^3$  cells/ cm<sup>2</sup> and  $10^4$  cells/ cm<sup>2</sup> and *MSX-2* expression was essentially undetectable, it is possible that the expression of *MSX-2* in these cells, and its induction by calcitriol, is dependent upon cell-cell communication.

Ascorbate-2-phosphate, a stable analogue of ascorbic acid, is required for the formation of a fully functional extracellular matrix by bone-derived cells cultured in vitro as it promotes post-translational modification of collagen (Franceschi and Iyer, 1992). Culturing cells in the absence of ascorbate-2-phosphate would result in little or no functional extracellular matrix, possibly affecting the differentiation ability of the

cells. Therefore, this would allow for the examination of the importance of cell:matrix interaction.

In these studies, it was shown that the responsiveness of cells to calcitriol was generally increased in the presence of an extracellular matrix. Basal and calcitriol-induced expression of MSX-2 was generally enhanced in the presence of an extracellular matrix: without ascorbate-2-phosphate, only a high dose of calcitriol could induce expression of this gene above steady state levels. Induction of osteocalcin expression by calcitriol is essentially unaffected by the absence or presence of extracellular matrix.

The induction of BMP-2 by calcitriol is dose-dependent in the absence of an extracellular matrix whereas with ascorbate-2-phosphate, BMP-2 expression is suppressed with calcitriol. This suggests that expression of this gene occurs in proliferating cells and that it is decreased in cells with a more differentiated phenotype. This suggests that induction of BMP-2, then its downregulation, may be a first stage in the progression from proliferation to commitment.

When comparing the effects of increasing doses of calcitriol in cells cultured in the absence or presence of an extracellular matrix, interesting patterns emerge. The biphasic effects of calcitriol on osteoblast-related gene expression in the presence of an extracellular matrix suggests that the extracellular matrix potentiates the induction by calcitriol of expression of genes which are thought to act earlier in the differentiation pathway, that is, BMP-2, BMP-4 and MSX-2, while having little effect on the induction of osteocalcin expression. This indicates that the extracellular matrix allows induction by calcitriol of some progress towards differentiation but has little effect on the terminal differentiation of these cells. In contrast, BMP-7-induced expression of osteocalcin is clearly enhanced in the presence of an extracellular matrix as compared to that seen in cells treated with the same doses of the agent in the absence of ascorbate-2-phosphate. Taken together, these results suggest that cell:matrix communication is agent-dependent and therefore will affect the pattern of gene expression and the progression through differentiation accordingly.

The temporal pattern of gene expression was investigated in cells cultured in the presence of ascorbate-2-phosphate and treated with 10 nM calcitriol over a period of 24 hours. The gene expression pattern in untreated cells at time= 30 minutes was used as control as the time= zero timepoint was lost. The results are difficult to interpret due to the replacement of medium which was coincident with treatment of the cells with calcitriol. Endogenous calcitriol may be present in the serum contained in the fresh medium and could have affected the outcome of the experiment. As noted in the results chapter, calcitriol treatment after 24 hours was seen to have little effect on the steady state levels of BMP-2, BMP-4 and osteocalcin and to stimulate MSX-2 levels. MSX-2 expression was marginally stimulated with calcitriol treatment initially and this

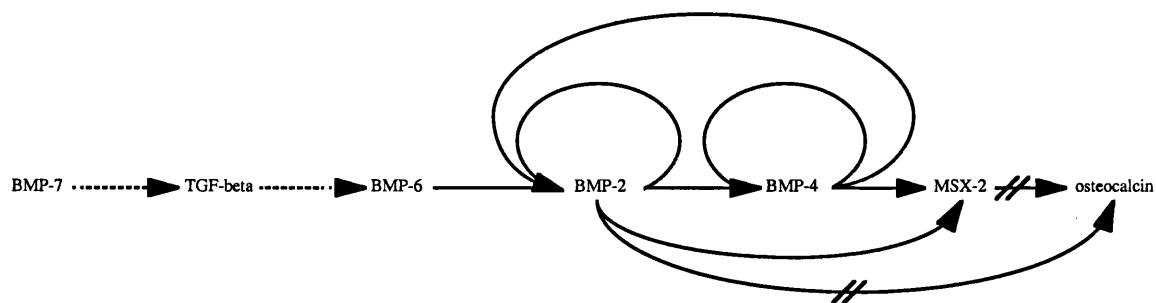
modest increase was steadily maintained throughout the course of the experiment to result in an overall rise in MSX-2 expression when compared with untreated cells at the start or at the endpoint of the experiment. The fresh serum may have maximally increased osteocalcin mRNA expression such that addition of exogenous calcitriol would have no further effect upon its expression. For this reason, all other experiments reviewed in this chapter were performed following replacement of medium, supplemented with 2% fetal calf serum, 24 hours prior to treatment with exogenous factors.

The effects of members of the TGF-beta superfamily on gene expression in trabecular bone-derived cells cultured in ascorbate-2-phosphate were examined. The inducibility of BMP-2 mRNA expression by TGF-beta and by all four of the BMPs tested suggests that it is expressed last. The inhibitory effects of BMP-2 on BMP-4 expression observed in MG-63 cells were also apparent here, but at a dose four times greater than in MG-63 cells: in trabecular bone-derived cells, only the maximal dose of BMP-2 ablated BMP-4 expression. BMP-4 simulates BMP-2 expression, and enhances its own. Therefore, at doses of 100 ng/ml or less there is positive regulation of BMP-4 mRNA expression by BMP-2. It would appear that different doses of BMPs are required to exact the same effects on their gene expression in osteoblastic cells as in MG-63 cells.

MSX-2 expression was enhanced by any dose of BMP-2, BMP-4 or BMP-6. BMP-7 treatment had a dose-dependent trend in increasing MSX-2 expression but several doses resulted in diminished MSX-2 mRNA levels. With BMP-6 treatment, levels of MSX-2 mRNA were increased dose-dependently. The inductive properties of BMP-6 are in keeping with the finding that BMP-6 affects earlier stage osteoprogenitor cells than either BMP-4 or BMP-2 (Hughes et al, 1995).

Treatment with BMP-4 or BMP-7 results in similar patterns of osteocalcin gene expression: essentially a dose-dependent increase is seen following treatment with either agent, with several doses exhibiting a less stimulatory effect. At doses of 5-100 ng/ml, BMP-7 has similar effects on BMP-4 expression as the same doses of BMP-4 have on osteocalcin expression. Taken together, this could indicate that BMP-7 action on BMP-4 expression may have a knock-on effect on osteocalcin expression. BMP-2 treatment induces osteocalcin expression at all doses. Treatment with BMP-6 stimulates osteocalcin maximally at low doses but the magnitude of this increase is reduced with increasing dose. This supports an implied role for BMP-6 in chondrogenic differentiation, where a transient increase and subsequent downregulation induces terminal differentiation in the absence of an osteogenic stimulus (Carey and Liu, 1995). At the dose tested, TGF-beta exerted the same stimulation in the expression of all four genes tested in osteoblastic cells as in MG-63 cells.

These data indicate a complex pattern of regulation of gene expression in trabecular bone-derived cells. While there are many similarities in the ability of agents to modulate the levels of osteoblast-related transcript expression, the relative potency of these factors differs from that seen in MG-63 osteosarcoma cells. In this cell line, it was seen that of the TGF-beta superfamily members, BMP-7 was the most potent of the factors tested, maximally increasing the expression of MSX-2, osteocalcin, BMP-2 and BMP-4 transcripts, whereas in trabecular bone-derived cells the most potent agent, also upregulating the mRNA expression levels of all four of the genes examined, was BMP-2.



A possible model for the cascade of osteoblast-related gene expression in human trabecular bone-derived cells

Overall, in MG-63 cells the increasing order of potency of factors in stimulating mRNA expression levels was BMP-2, TGF-beta and BMP-7. This order differs in trabecular bone-derived cells: in increasing order of potency, TGF-beta, BMP-7 and BMP-2 increase transcription of osteoblast-related genes. This may be due to the heterogeneity of the population of trabecular bone-derived cells: in each culture flask, cells which represent different stages in the differentiation pathway may be present, and the expression of transcripts within these cells may be modulated in a stage-specific, maturation-dependent manner, for example, in bone-derived cell populations, osteocalcin is expressed by the mature osteoblast phenotype only. It is therefore reasonable to suggest that the effects of bone-related factors may also be specific to the stage of differentiation of the cell. In relation to the cascade of osteoblast-related gene expression in the relatively undifferentiated MG-63 cell line, it can be inferred that the majority of cells in the trabecular bone-derived cultures are already partially differentiated and are at a stage when introduction of BMP-2 has the strongest effect. Another possibility is that the presence of the extracellular matrix, upon which factors may be sequestered, may have increased the local concentration of agents and elicited a different genetic response from that seen in the MG-63 cells, which secrete no matrix.

The concept of interactions between agents affecting the expression patterns of osteoblastic genes was examined in MG-63 cells in the first results chapter. This was investigated in trabecular bone-derived cells cultured in the presence of ascorbate-2-

phosphate, and the effects of fibroblastic growth factor-2 (FGF-2) alone or in combination were also observed. BMP-2 and MSX-2 expression was increased following treatment of the cells with any agent, alone or in combination. When compared to control, osteocalcin expression was shown to be augmented following treatment with members of the TGF-beta family in combination with each other or with calcitriol; in contrast, FGF-2, alone or in any combination with the other agents tested, either did not affect steady state levels of osteocalcin transcript expression, or decreased them. This strongly suggests that FGF-2 prevents the terminal differentiation of osteogenic cells. BMP-4 expression was most interestingly moderated by agents when added alone: calcitriol, BMP-2 and TGF-beta stimulated expression, and mRNA levels were unaffected following treatment with BMP-7 or FGF-2. FGF-2 or BMP-7 therefore maintain the basal expression of BMP-4 mRNA and perhaps mainly promote proliferation in cells at this early progenitor stage, whereas the other agents promote osteogenic differentiation. Of the combinations, BMP-7 plus TGF-beta had no effect, as perhaps the block on upregulation by BMP-7 prevented the stimulatory effects of TGF-beta. BMP-7 plus calcitriol plus or minus FGF-2, attenuated BMP-4 mRNA levels but strongly augmented those of osteocalcin which suggests that these combinations are markedly osteogenic.

The effects of these interactions are obviously complex, and of these only BMP-2 and FGF-2 or of FGF-2, calcitriol and BMP-7 in combination attenuates expression of BMP-4 and osteocalcin at the same time. This suggests that these particular combinations suppress the differentiation of the cells and may in fact encourage proliferation. However, the proliferation of these cells treated or untreated was not tested.

The interaction of calcitriol and FGF-2 stimulated expression of BMP-2, BMP-4 and MSX-2 and blocked terminal differentiation. This observation is of interest as the action of either agent alone had no effect on osteocalcin expression, and no effect on MSX-2 or BMP-4 mRNA expression respectively. A possible explanation is that this combination may have impeded the progress of differentiation and, with a longer treatment and an examination of the kinetics of the temporal pattern of expression following this combined treatment, this may have become apparent.

To investigate the mechanisms of regulation of gene expression in trabecular bone-derived cells, secondary passaged cells cultured in the presence of ascorbate-2-phosphate were treated with cycloheximide, actinomycin D and calcitriol, alone or in combination. The stimulation of the mRNA expression of all four genes of interest by calcitriol was noted. The enhancement of the expression levels of BMP-2, BMP-4 and MSX-2 following treatment with cycloheximide and calcitriol indicates that the regulation of their expression involves (an) intermediate labile protein(s). Osteocalcin

expression is essentially unaffected by cycloheximide treatment, supporting the direct regulation of the expression of this gene by calcitriol.

The recent finding that BMP-4 transcript expression is regulated by E-box proteins (Ebara et al, 1997) suggests a mechanism of regulation of BMP-4, BMP-2 and MSX-2 transcript expression. Inhibitor of DNA binding/differentiation (Id) proteins are helix-loop-helix transcription factors (HLH TFs) lacking the basic DNA binding domain. The HLH protein-protein binding domain is retained, allowing heterodimerisation with basic HLH TFs which prevents their binding to DNA, thus negatively regulating transcription (Simonson et al, 1993). Targets of Id binding include E-box proteins (Kreider et al, 1992), homeodomain-containing transcription factors which bind to the E-box motif CANNTG and activate transcription (Jen et al, 1992). In the 1 kb fragment immediately 5' to the coding sequence of the *Msx-2* gene (see figure 4.10), five such motifs exist. Also noted is the absence of a vitamin D response element. Calcitropic agents have been shown to modulate Id expression: calcitriol dose-dependently suppressed its expression (Kawaguchi et al, 1992). To be noted was that cycloheximide had no effect on the vitamin D inhibition of Id transcription, indicating that the effect of vitamin D on Id expression is a direct one (Ogata et al, 1993). Therefore it is possible that MSX-2 and BMP-4 mRNA expression is regulated by E-box proteins and, by conjecture, subject to modulation by Id proteins.

Treatment with actinomycin D downregulated transcript levels of BMP-4, had little effect on those of MSX-2 and osteocalcin, but increased those of BMP-2. This suggests that transcripts for BMP-4 have a high turnover rate and are therefore more unstable than those for the other genes examined. Also, as outlined previously, reduced levels of BMP-4 stimulate BMP-2 mRNA expression, possibly accounting for the increase in BMP-2 levels. It is possible that a longer exposure to a higher dose of actinomycin D would have resulted in a downregulation of the mRNA levels of the other genes of interest, however interpretation is complex in the absence of measurement of the half-lives of these mRNAs.

The conclusions of this chapter are that the regulation of osteoblast-related gene expression in osteoblastic cells is complex and has a pattern of expression closely resembling that seen in MG-63 osteosarcoma cells. In the case of trabecular bone-derived cells, BMP-2 is the most potent regulator of osteoblast-related gene expression. However, within the limitations of the examination of the effects of these agents on the expression of the genes tested, MG-63 cells were an adequate model of adult human osteoblastic differentiation. Cell-cell and cell-matrix communication were seen to be critical factors in determining the genetic response of osteoblastic cells to the agents. Regulation of the expression of these osteoblast-related genes is intricate and may involve interactions between osteoinductive agents sequestered in or immobilised



on bone. The calcitriol-induced upregulation of expression of the osteogenic commitment/differentiation genes BMP-4, BMP-2 and MSX-2 may require an additional, as yet unidentified, labile intermediate protein.

This results chapter shows that there are differences in the pattern of gene expression in osteoblasts, which exhibited some variation from one experiment to another. These differences and variations could be explained by the differences between donors, such as age, gender and site of extraction (see Appendix 2), or by the heterogeneity of cell populations within flasks, such that in any flask, there may be cells at different stages of maturation with different patterns of gene expression. Therefore, it would be of considerable interest to define these subsets of cells in such a way that gene expression patterns could be examined within each subpopulation separately.

```

-1233 gga tcctgatagg tatgctgctt aaaatttcaa agtCAGGTGa gatgagcttt ccccaaagat attcaggctt
-1160 tgggtttgca aagtcgaacc ctgttatttg cagagagaat aaaggcacta aatgtcacia atacccttct
-1090 cccctgccca tcacctccca agaCAGATGc tatgctgaCA CCTGaactec aatgacaaac
-1030 acaaactcaa aacaacaaaa caaacaacaa aaaaacctca tgagcacact ttgcacggg ctagagagg
- 960 agactcaagg ggtgagaaat atcccgattg acagtttcta aaactgtcc tgttggaca gtgtatgaaa
- 890 tggagatCAT TTGagtaaata tagctgccag aaccaatggg ggctgggtct gctgggtgcac acctacagtc
- 820 tgaacaccag ggacgaagcc agtggggtag caagttcagg accatcttgg gtaatatagt gagcgacttc
- 750 taagtagcc agagctggag agcaagagtc tgttttaaag gaaaaaaaaa aaggagagaaa gggtagagtt
- 680 agttgaatat ccttggcca cctctcagcc ttctcatct ttgtccctt tttaaggaa tgagttacag ctaatgtctt
- 600 ggaacactct ccaagataa gacaactaag gaccatgcct aaactatttc tattagtaaa gatgctgcta
- 530 ctgttttcca ttatagtaca aagatacact agtttgagct catagtggga gctttataaa tgttccatgc
- 460 cctccgcaga ttccaacat tctcaggcgg gagcgtgtgg gatcggcacc agaaacactt taaagagggg
- 390 ttaaaagaca agaaatgaga ctcaacaagc ttctcttta aacaatcggc ttaattacg ccttagactt
- 320 ttg/cagtttgg gcgattataa cccttagagg atcgccctaat aacaactctg ctgactgctc ctgtaattaa
- 250 ctctaattt atttcaaagc gggcggggga agggcccaa gcctctccag ggagagccaa tcggtggcga
- 180 gcgtccgtgg cgtcaggagc agggccgtcg cCAGTTGgtt gagccgagtc tcccacttc
- 120 cctcgagga gaggtgggc tccagcgcg ccctgccgg cccccccc aaaagtggga gtcttcgctt
- 50 gagagttgcc agcggagtcg gcgcccga gctacgcggc gcagaaagtc

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Figure 4.10 The 5' flanking sequence of the mouse msx-2 gene

The sequence above is the 1.2kb fragment immediately upstream of the coding sequence for the murine msx-2 gene. Indicated in bold type are the putative binding sites for E-box binding proteins.

## **Chapter 5**

**Effects of calcitriol and dexamethasone on the expression of osteoblast-related genes in subpopulations of human trabecular bone- and bone marrow-derived cells**

## Introduction

Glucocorticoid treatment is associated with decreased bone mass and a change in cell activity. Continued exposure to glucocorticoids can result in osteoporosis through stimulation of bone resorption and reduction of bone formation (Cosman et al, 1994). Effects of glucocorticoids on osteoblasts include suppressed replication of preosteoblasts and attenuation of type I collagen, osteocalcin expression and tissue inhibitor of metalloproteinases (TIMPs) (Doherty et al, 1995), although as osteoblastic differentiation is galvanised (reviewed by Delany et al, 1994) and mineralisation and bone nodule formation is stimulated by treatment of osteoblastic cell cultures with dexamethasone (Iba et al, 1995; Ishida et al, 1995), these effects must depend on the stage of maturation of the cell. Interaction of osteoblasts with their extracellular matrix is limited by glucocorticoid treatment: decreased  $\beta_1$ -integrin levels as a consequence of glucocorticoid treatment caused inhibition of attachment of osteoblasts to their extracellular matrix as this integrin is necessary for the binding of fibronectin and collagen (Gronowicz and McCarthy, 1995): fibronectin synthesis and secretion is also abrogated in cells treated with dexamethasone (Gronowicz et al, 1991). The premature detachment of osteoblasts from their extracellular matrix has clearly important implications for the development of osteoporosis.

As described previously, clonogenic progenitor cells (colony forming unit-fibroblastic cells (CFU-F)), which are associated with the marrow stroma, give rise to osteoblasts when treated with a differentiation stimulus (see Introduction, section 1.4). In the culture of adult human bone marrow cells, a high proportion of CFU-F can be induced to become osteoblastic following continuous treatment in the presence of a physiological dose of glucocorticoid, for example 10 nM dexamethasone (dex) (Cheng et al, 1994). Therefore, these cells, cultured in the presence or absence of dex and other factors, can be used in an attempt to investigate more fully the differentiation stage-specific expression of osteoblast-related genes.

Variations in the induction of gene expression by growth factors observed in trabecular bone-derived cells seen in the previous chapter may be due to differences in donor age, sites of extraction or due to the presence of cells at different stages of differentiation within the cultured cell populations. There is good evidence (Stewart et al, 1996) to suggest that expression of the Stro-1 and alkaline phosphatase cell surface antigens may reflect the maturity of the osteoblast. The expression of Stro-1 occurs earlier than, but later overlaps with, that of alkaline phosphatase, with expression of both markers being downregulated in sequence. This results in a progression from the double negative state (Stro-1 negative, alkaline phosphatase negative) to Stro-1 positivity (multipotent precursor), to double positivity (committed osteoprogenitor), to alkaline phosphatase positivity alone (mature osteoblast), to a double negative state again. The double negative subpopulation is the least well defined in that it may contain Stro-1 negative, alkaline phosphatase negative precursors, which can acquire positivity for both these antigens, as well as late stage cells of the osteoblast lineage, such as osteocytes and lining cells, which

have downregulated expression of these antigens. Nevertheless, when used in conjunction, the Stro-1 and alkaline phosphatase cell surface antigens can be used to “stage” the early differentiation of adult human cells of the osteoblast lineage. It therefore seemed reasonable to examine the expression of osteoblastic genes within these separate subpopulations of trabecular bone- and bone marrow-derived cells and to investigate the effects of calcitriol on gene expression within these fractions.

In the following chapter, the subpopulations are denoted thus:

R2: Stro-1 antibody negative (Stro-1 -ve), alkaline phosphatase antibody negative (AP-ve);  
 R3: Stro-1 antibody positive (Stro-1 +ve), alkaline phosphatase antibody negative (AP-ve);  
 R4: Stro-1 antibody positive (Stro-1 +ve), alkaline phosphatase antibody positive (AP+ve);  
 R5: Stro-1 antibody negative (Stro-1 -ve), alkaline phosphatase antibody positive (AP+ve).

As well as further investigating the results of calcitriol treatment, the effects of the glucocorticoid, dexamethasone (dex), on osteoblast-related gene expression and on the distribution of cells between the four subpopulations was investigated.

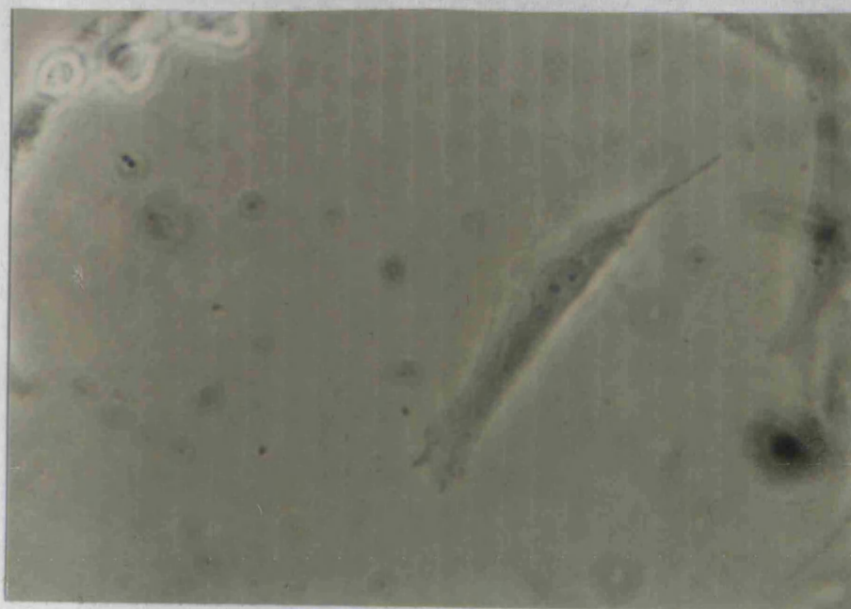
Either the R3 or R4 subpopulation was used to establish a ratio in the course of these experiments as occasionally not all subpopulations were represented. Asp was present constantly in all cultures. All PCRs and Southern blots were repeated twice, with each experiment repeated up to four times.

### 5.1 The effects of constant culture in dex on the morphology of adult human bone marrow-derived cells

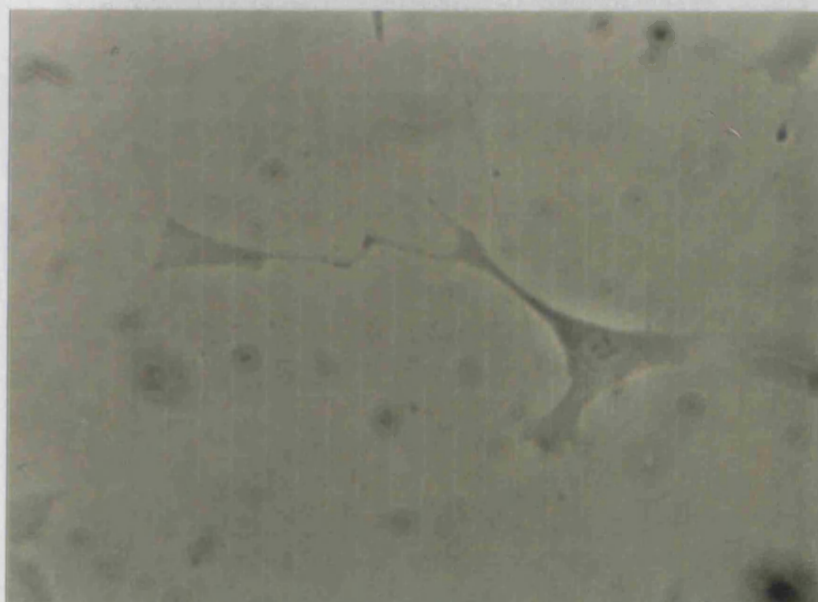
Figure 5.1 illustrates the effects of dex on cell morphology (photograph 5B) as compared to cells cultured in the absence of dex (photograph 5A): untreated cells tend to have an elongated appearance whereas those treated with dex are more polygonal in shape.

### 5.2 Sorting subpopulations of human bone marrow-derived cells according to their expression of the Stro-1 and alkaline phosphatase cell surface antigens

First passage bone marrow-derived cells were maintained in DMEM containing 10% fetal calf serum and 100  $\mu$ M asp and in the absence or presence of 10 nM dex until confluent at which point they were cultured for 24 hours in DMEM supplemented with 2% fetal calf serum and 100  $\mu$ M asp, and in the absence or presence of dex as appropriate. Cells were then exposed to 10 nM calcitriol for 24 hours. Following labelling for the cell surface expression of Stro-1 and alkaline phosphatase, cells were separated by FACS and the fractions retrieved for analysis. If sufficient cells were collected, the purity of subpopulations was estimated by performing FACS analysis again: only those subpopulations of at least 85% purity were analysed for mRNA transcript levels of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting.



Photograph 5A First passage human bone marrow-derived cell cultured in the absence of dexamethasone. This was photographed at x320 magnification.



Photograph 5B First passage human bone marrow-derived cell cultured in the presence of 10 nM dexamethasone. This was photographed at x320 magnification.

Figure 5.1 The effects of constant culture in dexamethasone on the morphology of adult human bone marrow-derived cells

First passage bone marrow-derived cells were plated at a density of  $5 \times 10^3$  cells/  $\text{cm}^2$  and were maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu\text{M}$  ascorbate-2-phosphate in the absence (5A) or presence of 10 nM dexamethasone (5B). Untreated cells display a typical elongated fibroblastic appearance whereas those maintained in the presence of dexamethasone exhibit a more truncated, polygonal appearance.

The results of one experiment of treatment with dex and calcitriol on the distribution of cells within the four subpopulations separated according to their expression of Stro-1 and AP are detailed in figures 5.2a- d. For each figure, there are five dot plots: one showing secondary labelling, labelling with Stro-1 or AP only, and labelling for both Stro-1 and AP, and finally a plot showing the gating for subpopulations, with R2 in the lower left quadrant and moving clockwise to R5 (lower right quadrant). Table 5.1 shows the distribution of cells treated with calcitriol and/ or dex between the four fractions of four FACS-analysed experiments as compared with the distribution of control cells.

Under the culture conditions used in these experiments, calcitriol modestly decreased the proportion of cells present in the R2 and R3 regions and increased that in the R4 and R5 subpopulations. Continuous culture in the presence of dex more strongly reduced the proportion of cells present in the R2 and R3 fractions and increased the proportion of cells present in the R4 and R5 fractions. The effects of treatment of bone marrow-derived cells with calcitriol and dex in combination were essentially similar to those seen in cultures treated with dex alone

As well as examining the number of cells expressing Stro-1 and/ or AP in the population of cultured cells, it is possible to determine the level of expression of these cell surface antigens by measuring their mean fluorescence intensity (MFI) in the FL-1 (AP) and FL-2 (Stro-1) channels (X geo mean and Y geo mean respectively). Data for the MFIs obtained from four independent experiments were pooled and summarised in table 5.2. When compared with control, the level of expression of the Stro-1 antigen was not affected following treatment with calcitriol. However, following treatment with dex alone or when added in combination with calcitriol, there was a tendency towards a reduction in Stro-1 antigen expression in the Stro-1 positive subpopulations (R3 and R4 fractions). Treatment with calcitriol alone increased the level of AP antigen in both the AP positive subpopulations (R4 and R5 fractions). The effects of dex were similar, but of greater magnitude. When added in combination with dex, calcitriol antagonised the stimulatory effects of dex on the levels of expression of this enzyme.

### 5.3 The effects of calcitriol and dex, alone or in combination, on the expression of osteoblast-related genes in subpopulations of bone marrow-derived cells

First passage bone marrow-derived cells were maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M asp and in the absence or presence of 10 nM dex until confluency when the cells were cultured for 24 hours in DMEM containing 2% fetal calf serum and 100  $\mu$ M asp and 10 nM dex as appropriate. The cells were then exposed to 10 nM calcitriol for 24 hours. RNA was harvested and analysed for the mRNA expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting (figures 5.3a and 5.3b). The PCRs and blots were carried out twice and average values following densitometric analysis were taken. This experiment was performed three times and, in addition, the cDNA from another donor treated only with dex was supplied for

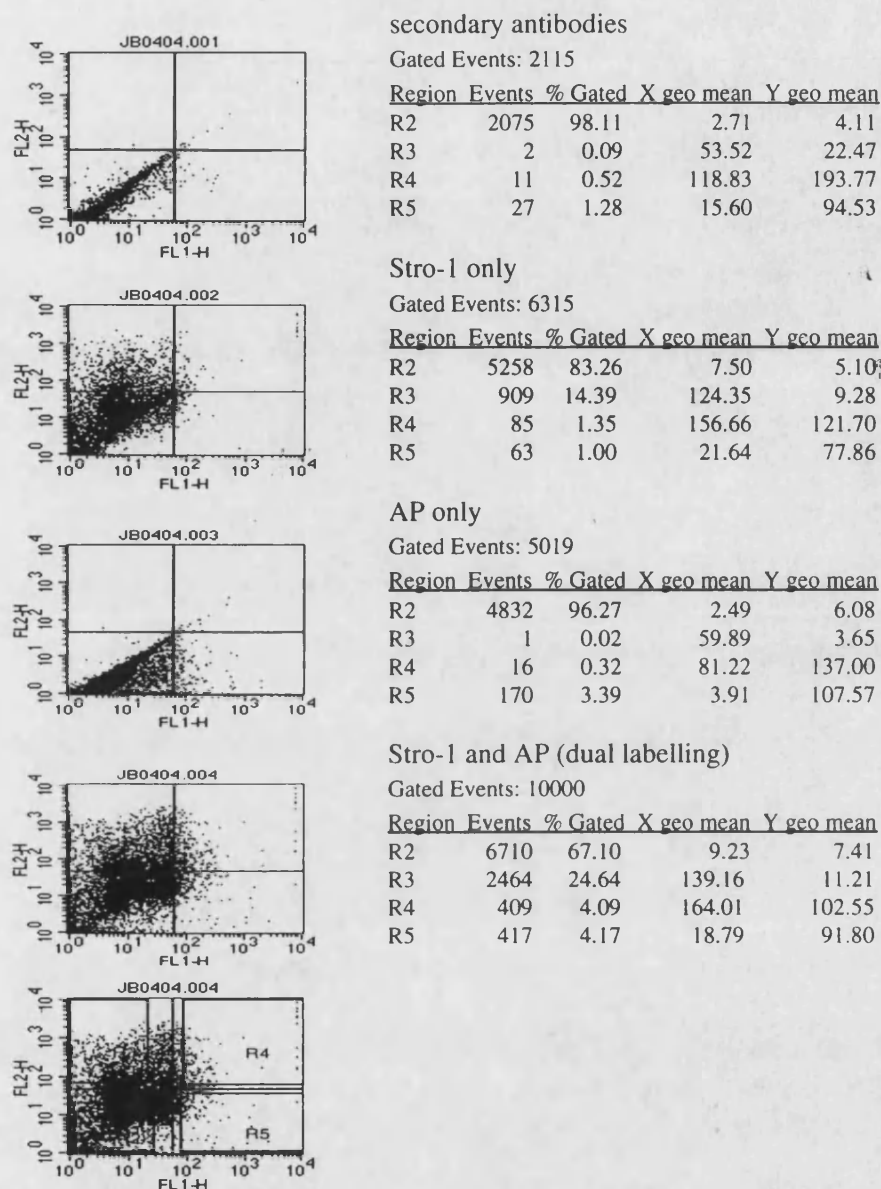
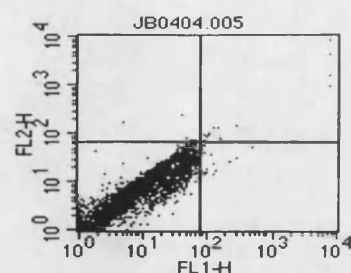


Figure 5.2a Sorting subpopulations of human bone marrow-derived cells according to their expression of the Stro-1 and alkaline phosphatase cell surface antigens

Confluent first passage human bone marrow-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 48 hours in DMEM containing 2% fetal calf serum and ascorbate. Cells were labelled with mouse anti-human antibodies raised against Stro-1 (IgM) and alkaline phosphatase (IgG<sub>1</sub>), then with goat anti-mouse secondary antibodies conjugated to the fluorochromes, FITC and R-PE. Cells were then separated according to the expression of Stro-1 and alkaline phosphatase by the use of fluorescence activated cell sorting (FACS).

The results are presented as dot plots: in the example above, in each graph any dot above or to the right of the central plot axis corresponds to positive labelling for Stro-1 and alkaline phosphatase (AP) respectively. Therefore the regions R2-R5 correspond to double negative, Stro-1 positive alone, positive for both antibodies and positive for AP only. Percentages of cells within each subpopulation, and the mean fluorescence intensity of the labelling of cells within each subpopulation, are recorded in tables to the right of each graph.

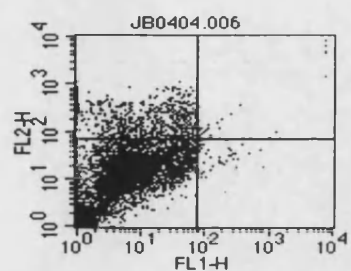




## secondary antibodies

Gated Events: 5025

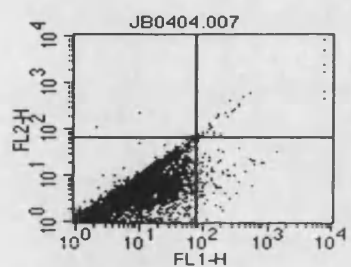
Region	Events	% Gated	X geo mean	Y geo mean
R2	4969	98.89	5.41	6.99
R3	11	0.22	86.95	41.15
R4	21	0.42	506.11	702.93
R5	24	0.48	30.65	113.59



## Stro-1 only

Gated Events: 6512

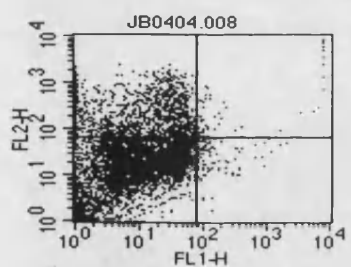
Region	Events	% Gated	X geo mean	Y geo mean
R2	5640	86.81	9.74	5.98
R3	788	12.10	160.41	11.35
R4	35	0.54	245.38	293.59
R5	179	2.75	29.63	137.15



## AP only

Gated Events: 6763

Region	Events	% Gated	X geo mean	Y geo mean
R2	5715	84.50	3.01	5.16
R3	124	1.83	21.48	22.55
R4	278	4.11	36.83	60.75
R5	646	9.55	6.33	46.39



## Stro-1 and AP (dual labelling)

Gated Events: 7115

Region	Events	% Gated	X geo mean	Y geo mean
R2	6621	97.90	3.59	6.78
R3	5	0.07	95.77	22.39
R4	34	0.50	221.08	316.14
R5	103	1.52	8.48	135.86

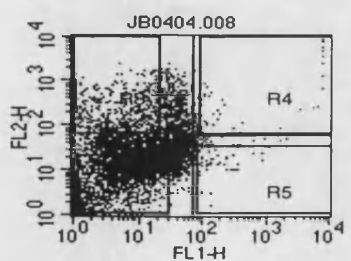
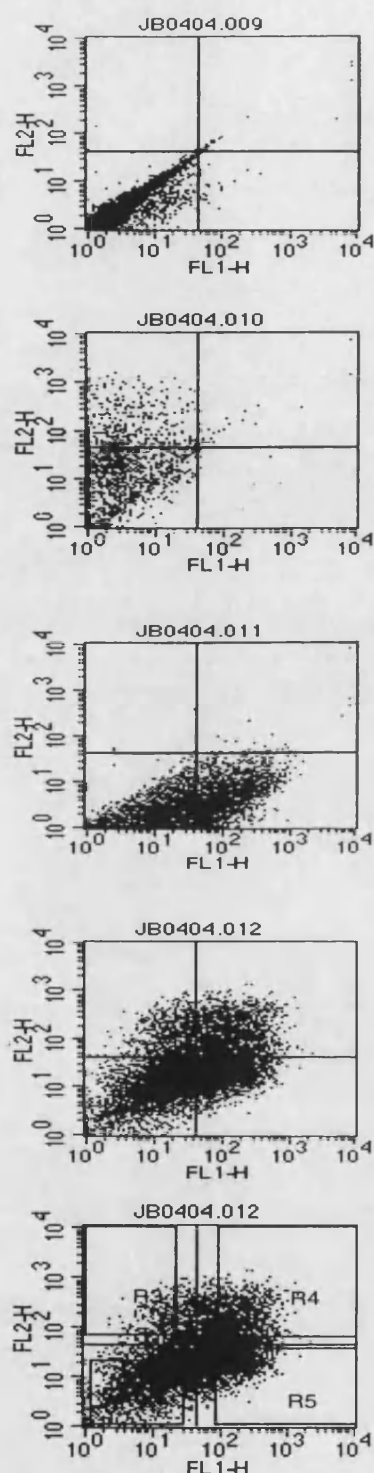


Figure 5.2b The effects of calcitriol on the expression of the Stro-1 and alkaline phosphatase cell surface antigens on human bone marrow-derived cells

Confluent first passage human bone marrow-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. Cells were then exposed to 10 nM calcitriol for 24 hours before being labelled for the expression of Stro-1 and AP cell surface antigens as previously described.





### secondary antibodies

Gated Events: 5102

Region	Events	% Gated	X geo mean	Y geo mean
R2	5044	98.86	2.64	3.18
R3	4	0.08	57.90	6.87
R4	30	0.59	100.75	142.44
R5	24	0.47	9.52	70.49

### Stro-1 only

Gated Events: 1980

Region	Events	% Gated	X geo mean	Y geo mean
R2	1533	77.42	5.76	2.45
R3	416	21.01	128.82	4.29
R4	26	1.31	140.80	129.00
R5	5	0.25	28.54	75.26

### AP only

Gated Events: 5025

Region	Events	% Gated	X geo mean	Y geo mean
R2	2942	58.55	1.99	9.92
R3	6	0.12	67.12	8.98
R4	28	0.56	85.46	438.13
R5	2049	40.78	4.85	123.58

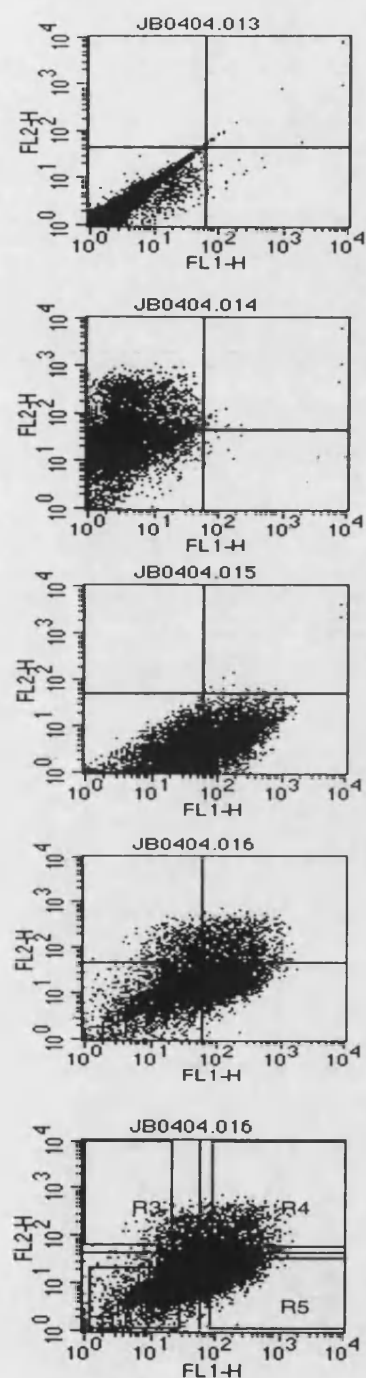
### Stro-1 and AP (dual labelling)

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	4416	44.16	10.10	12.01
R3	967	9.67	112.04	19.88
R4	2011	20.11	121.22	142.54
R5	2606	26.06	18.58	111.72

Figure 5.2c The effects of dexamethasone on the expression of the Stro-1 and alkaline phosphatase cell surface antigens on human bone marrow-derived cells

Confluent first passage human bone marrow-derived cells maintained in DMEM supplemented with 10% fetal calf serum, 100  $\mu$ M ascorbate-2-phosphate and 10 nM dexamethasone were cultured for 48 hours in DMEM containing 2% fetal calf serum and ascorbate and dexamethasone. Cells were labelled for the expression of Stro-1 and AP cell surface antigens as previously described.



### secondary antibodies

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	9951	99.51	2.79	3.38
R3	8	0.08	50.77	56.55
R4	22	0.22	134.88	192.66
R5	19	0.19	10.94	122.63

### Stro-1 only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	7145	71.45	14.44	2.94
R3	2820	28.20	128.46	5.08
R4	24	0.24	110.48	146.07
R5	11	0.11	20.45	93.74

### AP only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	5152	51.52	2.28	18.15
R3	0	0	0	0
R4	28	0.28	77.59	308.20
R5	48.20	48.20	5.53	160.28

### Stro-1 and AP (dual labelling)

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	4535	45.35	9.05	17.47
R3	671	6.71	106.16	28.53
R4	1557	15.57	113.41	191.95
R5	3237	32.37	18.18	143.91

Figure 5.2d The effects of calcitriol and dexamethasone in combination on Stro-1 and alkaline phosphatase cell surface antigen expression on human bone marrow-derived cells

Confluent first passage human bone marrow-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. Cells were then exposed to 10 nM calcitriol for 24 hours prior to labelling for the expression of Stro-1 and AP cell surface antigens as previously described.

Subpopulation Factor	R2 (-/-)	R3 (+/-)	R4 (+/+)	R5 (-/+)
untreated	55 (42-67)*	9.1 (6.3-25)*	3.7 (0-9.2)*	26 (3.2-42)*
calcitriol	38 (26-76)*	6.4 (3.5-21)*	7.5 (0.7-11)*	41 (1-57)*
dexamethasone	16 (0.5-44)*	1.8 (0-9.6)*	26 (20-51)*	48 (26-56)*
dexamethasone + calcitriol	13 (5.5-45)~	1.6 (1.4-6.7)~	25 (15-37)~	54 (32-56)~

Table 5.1 The effects of calcitriol and dexamethasone, alone and in combination, on the number of cells expressing the Stro-1 and/ or AP cell surface antigens in cultures of human bone marrow-derived cells

First passage bone marrow-derived cells maintained under standard culture conditions in the absence or presence of 10 nM dexamethasone were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate, and dexamethasone as appropriate. Cells were then exposed to 10 nM calcitriol for 24 hours. The cells were then labelled for expression of Stro-1 and AP cell surface antigens as previously described and were accordingly separated using FACS analysis. The table above summarises the proportions of cells distributed between the four subpopulations following treatment with calcitriol and/ or dexamethasone as compared to untreated cells. ~Value is the median from three independent experiments with the range shown in parentheses. \*Value is the median from four independent experiments with the range shown in parentheses.

Mean fluorescence intensity of Stro-1

<u>Subpopulation</u>	<u>R3 (+/-)</u>	<u>R4 (+/+)</u>
Factor		
untreated	139 (70-175)*	102 (73-164)*
calcitriol	127 (43-202)§	106 (42-256)§
dexamethasone	89 (62-142)*	61 (51-121)*
dexamethasone + calcitriol	80 (74-106)§	83 (67-113)§

Mean fluorescence intensity of AP

<u>Subpopulation</u>	<u>R4 (+/+)</u>	<u>R5 (-/+)</u>
Factor		
untreated	100 (68-193)*	96 (68-208)*
calcitriol	158 (54-250)§	135 (48-185)*
dexamethasone	258 (100-532)*	179 (85-275)*
dexamethasone + calcitriol	192 (183-565)§	146 (144-235)§

Table 5.2 The effects of calcitriol and dexamethasone, alone and in combination, on the level of expression of Stro-1 and AP cell surface antigens on subpopulations of adult human bone marrow-derived cells

First passage bone marrow-derived cells maintained under standard culture conditions in the absence or presence of 10 nM dexamethasone were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate, and dexamethasone as appropriate. Cells were exposed to 10 nM calcitriol for 24 hours. The cells were then labelled for expression of Stro-1 and AP cell surface antigens as previously described and were accordingly separated using FACS analysis. The top table summarises the proportions of the mean fluorescence intensity of labelling for Stro-1, and the bottom table represents those for AP between the four subpopulations following treatment with calcitriol and/ or dexamethasone as compared to untreated cells. § Value is the median from three independent experiments. \* Value is the median from four independent experiments, with the range shown in parentheses.

analysis by Joanne Screen, B.I.R.D. The median values, with the range of values shown in parentheses, were then entered into a table to facilitate comparisons between groups (table 5.3).

In the untreated cells, MSX-2 transcripts are expressed at essentially similar levels between subpopulations. Treatment with calcitriol or dexamethasone strongly increased the levels of mRNA for MSX-2 in the R2 and R3 subpopulations, whereas combined treatment resulted in an increase in MSX-2 transcript expression that was additive and supra-additive in the R5 and R2 subpopulations respectively, but in R3, the combined effects of these agents were no different from the effects of treatment with either agent alone.

Osteocalcin mRNA expression was lower in the R2 and R3 subpopulations of untreated cells when compared to R4. Calcitriol treatment stimulated levels in R2 and dexamethasone alone increased them in the R2 and R3 subpopulations. Calcitriol antagonised the stimulatory effects of dex on the expression of osteocalcin transcripts in cells from regions R2 and R3.

In untreated cells, BMP-2 transcript expression was minimal in the R2 and maximal in the R5 subpopulations when compared with R4. Treatment with calcitriol stimulated the levels of this transcript in the R2 and R3 regions, but decreased those in the R5 subpopulation. Dex alone strongly increased BMP-2 mRNA levels in the R2 and R3 regions. Addition of calcitriol and dex stimulated BMP-2 mRNA expression in R2 to levels comparable with the effects of either agent alone, whereas in R3 and R5, calcitriol antagonised the stimulation of BMP-2 transcript expression by dex to levels greater than those seen following treatment with calcitriol alone, when compared with R4.

BMP-4 transcripts were most strongly expressed in the R2 and R3 regions of untreated cells, when compared with R4. Treatment with calcitriol had essentially no effect on the levels of expression of BMP-4 transcript when compared to untreated cells. Dex alone stimulated BMP-4 transcript levels in the R5 and R2 regions but had no effect on levels in R3, when compared with R4. Treatment with calcitriol and dex in combination stimulated BMP-4 mRNA expression in R2 to levels comparable with the effects of dex alone and had a supra-additive effect in the R3 region, whereas in the R5 fraction, calcitriol antagonised the stimulation of BMP-4 transcript expression by dex to levels comparable with those in R4.

Statistical analysis was performed to compare gene expression patterns between the four subpopulations and can be seen in null hypothesis 23, Appendix 3.

#### 5.4 The effects of constant culture in dex on the morphology of adult human trabecular bone-derived cells

Figure 5.4 shows the effects of dex on cell morphology (photograph D) as compared to cells cultured in the absence of dex (photograph C): as observed in the

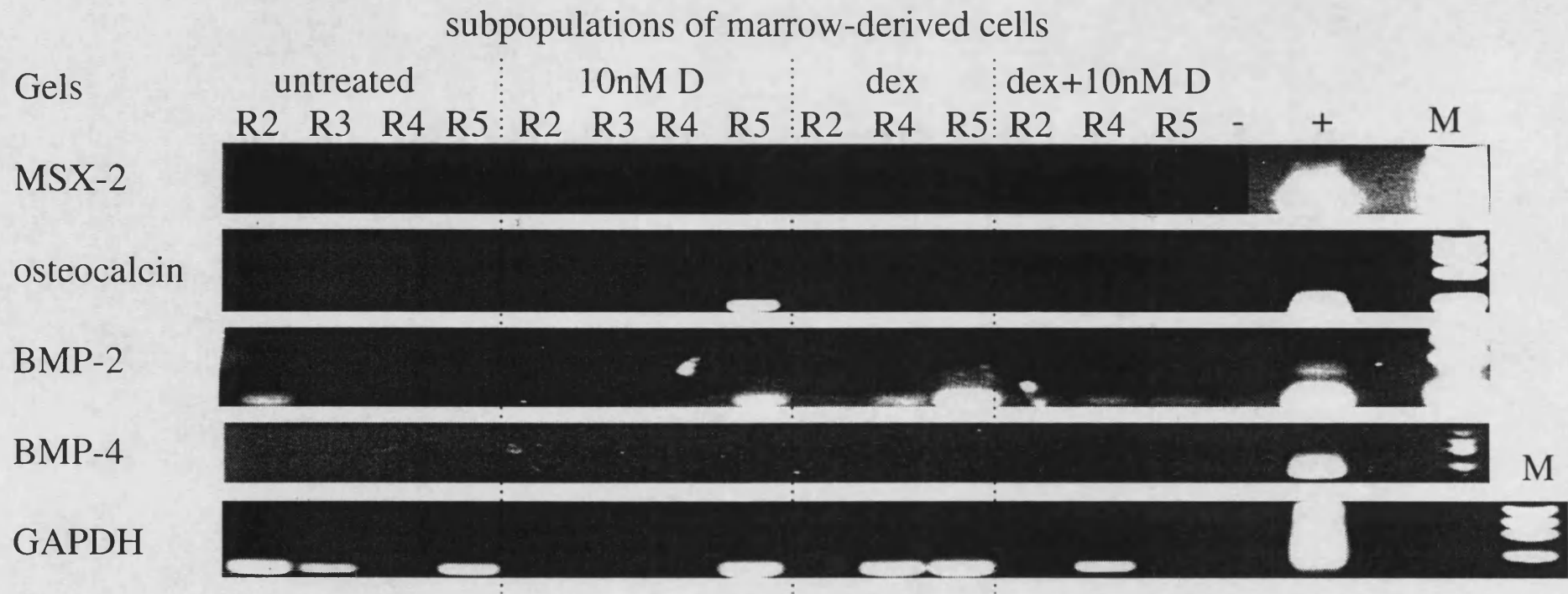


Figure 5.3a The effects of calcitriol and dexamethasone, alone or in combination, on the expression of osteoblast-related genes in subpopulations of adult human bone marrow-derived cells

Confluent first passage adult human bone marrow-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate, and in the absence or presence of 10 nM dexamethasone, were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate, and dexamethasone as appropriate. The cells were then exposed to 10 nM calcitriol for 24 hours prior to labelling for expression of Stro-1 and AP cell surface antigens. Accordingly, cells were separated into four subpopulations. RNA was extracted and analysed for the expression of mRNA transcripts for MSX-2, osteocalcin, BMP-2, BMP-4 (40 cycles) and GAPDH (35 cycles) by RT-PCR and Southern blotting. PCR and Southern blotting was performed twice for each gene and densitometry was carried out on the blots. Values obtained for direct repeats were averaged and median values, with their range shown in parentheses, were recorded in table 5.3. The gels above are representative of four independent experiments.

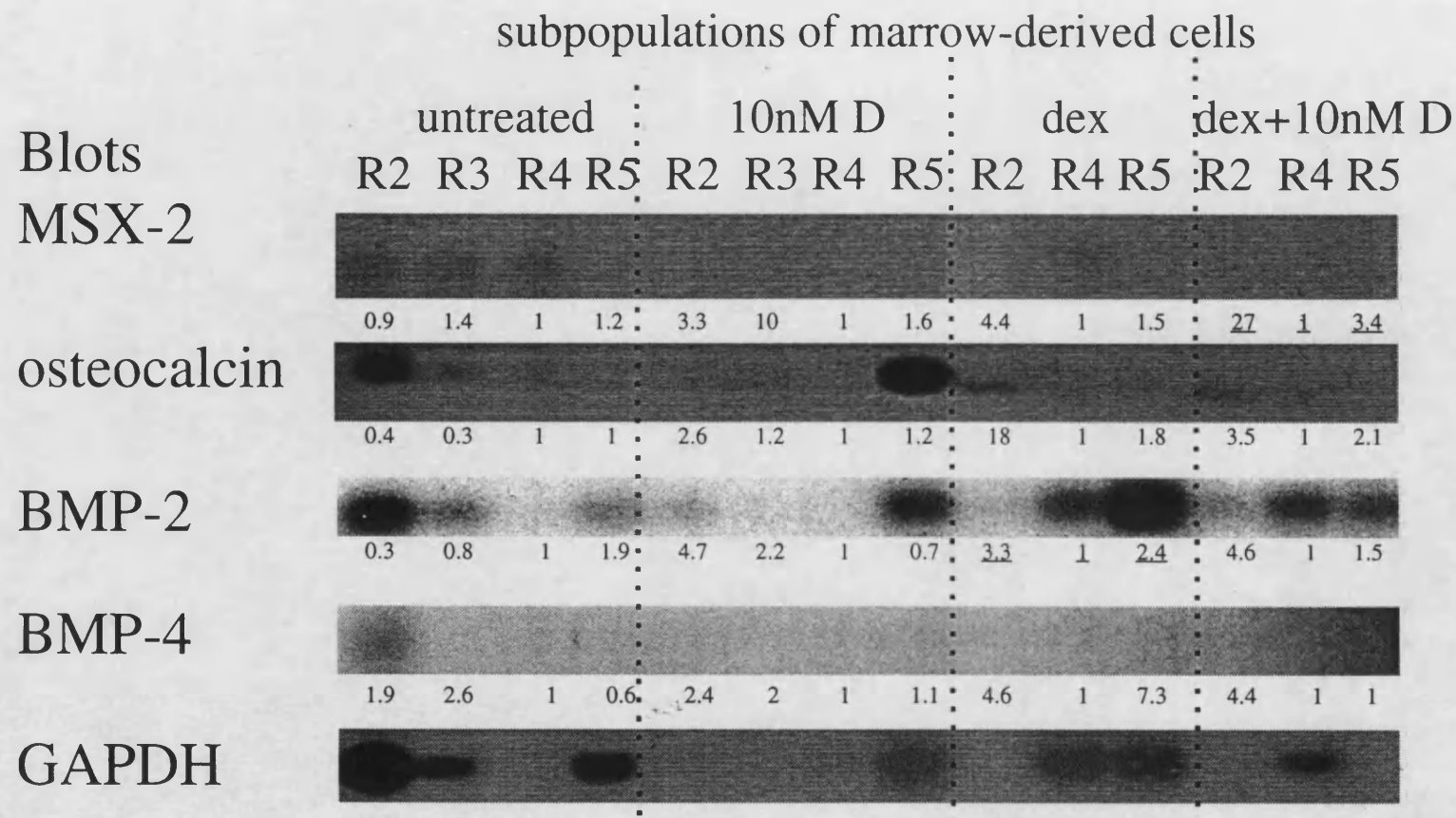


Figure 5.3b The effects of calcitriol and dexamethasone, alone or in combination, on the expression of osteoblast-related genes in subpopulations of human bone marrow-derived cells

This figure shows Southern blots for the experiment in figure 5.3a and is representative of four independent experiments. Densitometric values are shown beneath each blot - underline indicates statistical significance with H values.

Subpopulations		R2 (-/-)		R3 (+/-)	R4 (+/+)	R5 (-/+)
Factor	10 nM calcitriol	10 nM dexamethasone				
mRNA						
MSX-2	-	-	0.9 (0.3-1.6)†	1.4 (0.4-2.5)~	1†	1.2 (0.6-1.7)*
		+	4.4 (0.4-11)*	5.1 (0.3-10)~	1†	1.5 (1.5-1.5)~
		-	3.3 (2.9-11)†	10 (1.9-50)*	1†	1.6 (1.2-1.9)~
	+	+	27 (20-33)~	10≈	1*	3.4 (1.8-25)*
osteocalcin	-	-	0.4 (0.2-0.7)~	0.3 (0.3-0.4)~	1*	1 (0.9-1.1)~
		+	18 (6.3-30)~	10≈	1*	1.8 (1.7-2)~
		-	2.6 (0.7-4.5)~	1.2 (0.3-2.3)†	1†	1.2 (0.7-1.7)~
	+	+	3.5 (1-6)~	1.1≈	1*	2.1 (0.7-3.6)~
BMP-2	-	-	0.3 (0.2-41)†	0.8 (0.5-3.3)*	1†	1.9 (0.5-19)†
		+	3.3 (1.6-10)*	26 (1.3-50)~	1†	2.4 (1.5-7)†
		-	4.7 (0.4-9)~	2.2 (1.7-2.7)~	1†	0.7 (0.7-0.7)~
	+	+	4.6 (0.4-8.9)~	10≈	1*	1.5 (0.6-16)*
BMP-4	-	-	1.9 (0.3-40)*	2.6 (0.4-5)*	1†	0.6 (0.5-14)*
		+	4.6 (1.7-31)*	1.6 (0.8-2.5)~	1†	7.3 (3.2-12)*
		-	2.4 (0.9-3.9)~	2 (1.8-5)*	1†	1.1 (0.8-1.5)~
	+	+	4.4 (2-6.8)~	10≈	1*	1 (0.9-48)*

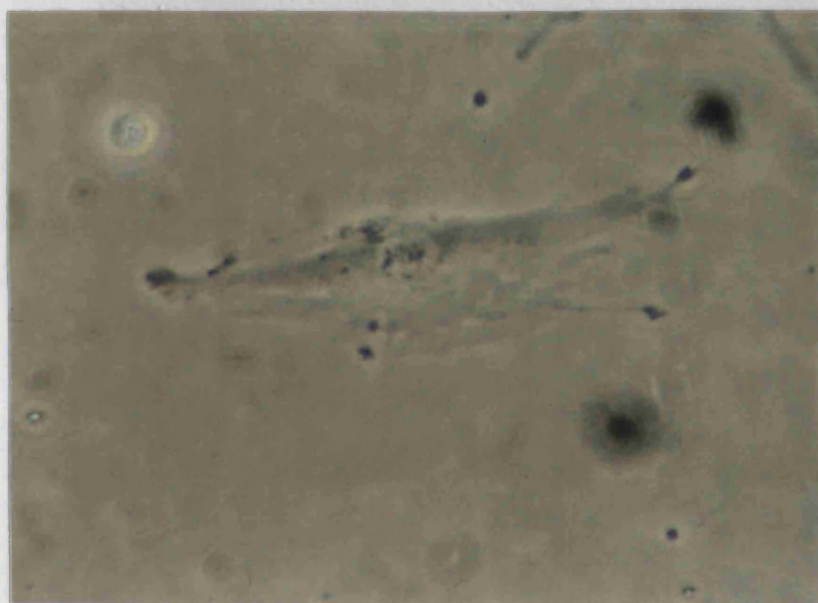
Table 5.3 The effects of calcitriol and dexamethasone, alone or in combination, on the expression of osteoblast-related genes in subpopulations of adult human bone marrow-derived cells

Confluent cells maintained under standard culture conditions and in the absence or presence of dexamethasone were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate, and dexamethasone as appropriate. The cells were treated with 10 nM calcitriol for 24 hours and then were harvested and labelled for the cell surface expression of Stro-1 and AP antigens. Cells were accordingly separated using FACS analysis. RNA was extracted from each subpopulation and examined for the mRNA expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting. These latter two processes were performed twice for each independent experiment. Densitometry was performed and the values obtained for repeats of PCRs and Southern blots were averaged. The table above represents the median values obtained from four independent experiments. ≈Value obtained from one experiment. ~Average value from two experiments; \* average value from three experiments; † average value from four experiments, with the range of values shown in parentheses.





Photograph 5C First passage human trabecular bone-derived cell cultured in the absence of dexamethasone. This was photographed at x320 magnification.



Photograph 5D First passage human trabecular bone-derived cell cultured in the presence of 10 nM dexamethasone. This was photographed at x320 magnification.

Figure 5.4 The effects of constant culture in dexamethasone on the morphology of adult human trabecular bone-derived cells

First passage trabecular bone-derived cells were plated at a density of  $5 \times 10^3$  cells/cm<sup>2</sup> and were maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate in the absence (5C) or presence of 10 nM dexamethasone (5D). Untreated cells display a typical elongated fibroblastic appearance whereas those maintained in the presence of dexamethasone exhibit a more truncated, polygonal appearance and seem to have more attachments to the plastic flask.

marrow-derived cells (see figure 5.1), untreated cells tend to have an elongated appearance whereas those treated with dex are more polygonal in shape.

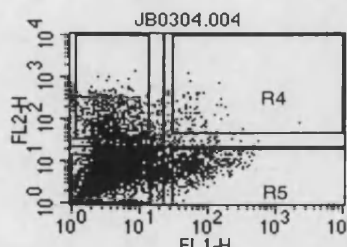
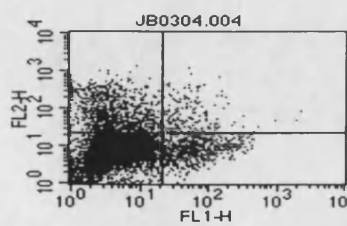
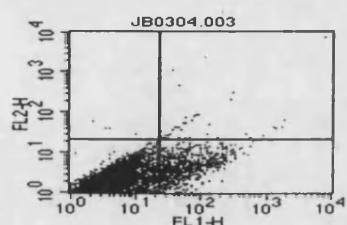
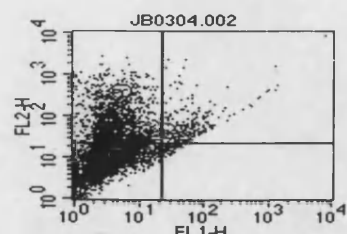
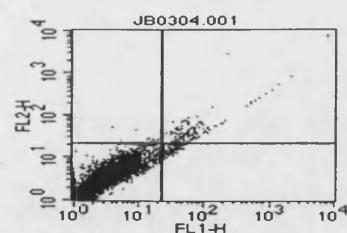
### 5.5 Sorting subpopulations of human trabecular bone-derived cells according to their expression of the Stro-1 and alkaline phosphatase cell surface antigens

First passage trabecular bone-derived cells were maintained in DMEM containing 10% fetal calf serum and 100  $\mu$ M asp and in the absence or presence of 10 nM dex until confluent at which point they were cultured for 24 hours in DMEM supplemented with 2% fetal calf serum and 100  $\mu$ M asp, and in the absence or presence of dex as appropriate. Cells were then exposed to 10 nM calcitriol for 24 hours. Following labelling for the cell surface expression of Stro-1 and alkaline phosphatase, cells were separated by FACS and where possible, the fractions retrieved for analysis of purity: those fractions showing greater than 85% purity were analysed for mRNA transcript levels of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting.

The results of one experiment of treatment with dex and calcitriol on the distribution of cells within the four subpopulations separated according to their expression of Stro-1 and AP are detailed in figures 5.5a- d. For each figure, there are five dot plots: one showing secondary labelling, labelling with Stro-1 or AP only, and labelling for both Stro-1 and AP, and finally a plot showing the gating for subpopulations, with R2 in the lower left quadrant and moving clockwise to R5 (lower right quadrant). Table 5.4 shows the distribution of cells treated with calcitriol and/ or dex between the four fractions of two FACS-analysed experiments relative to the distribution of control cells.

Under these culture conditions, calcitriol appears to have little effect on the expression of the Stro-1 and AP cell surface antigens in the R2 and R3 sort regions when compared to control cells, but decreases the proportion of cells present in the R4 region and modestly increases that in the R5 subpopulation. Continuous culture in the presence of dex strongly reduced the proportion of cells present in the R2 and R3 fractions and clearly increased the proportion of cells in the R4 and R5 fractions. The effects of treatment with calcitriol and dex in combination were similar to those seen following treatment with dex alone.

Table 5.5 shows the mean fluorescence intensity values for Stro-1 and AP labelling for the four fractions of cells following treatment with calcitriol and dex, alone and in combination for two FACS-analysed experiments. When compared with control, treatment with calcitriol or dex alone decreased the levels of Stro-1 antigen expression in the R4 fraction. Calcitriol and dex in combination produced levels of Stro-1 antigen expression in the R4 fraction comparable to those seen in cells treated with dex alone. Calcitriol markedly decreased alkaline phosphatase antigen expression in the R4 subpopulation when compared to control. Dex modestly decreased AP antigen expression in R4 and markedly stimulated it in the R5 region. Calcitriol modestly antagonised the effects of dex on AP antigen expression in the R4 and R5 subpopulations.



Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	9722	97.22	3.45	3.29
R3	17	0.17	26.25	13.03
R4	142	1.42	48.74	73.44
R5	119	1.19	14.67	31.28

Stro-1 only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	7639	76.39	8.02	3.34
R3	1998	19.98	62.09	4.71
R4	289	2.89	60.89	57.62
R5	74	0.74	15.70	30.73

AP only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	9021	90.21	2.15	3.93
R3	4	0.04	40.86	4.04
R4	41	0.41	53.01	97.36
R5	934	9.34	4.15	53.76

Stro-1 and AP (dual labelling)

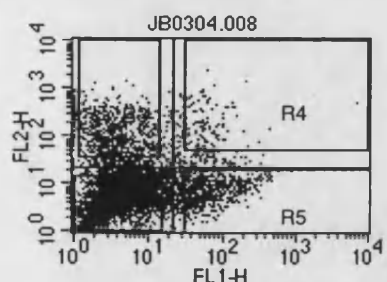
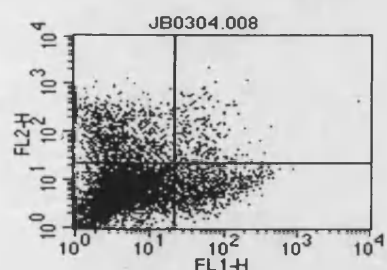
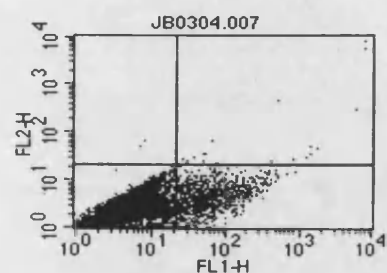
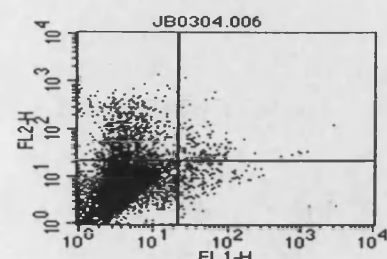
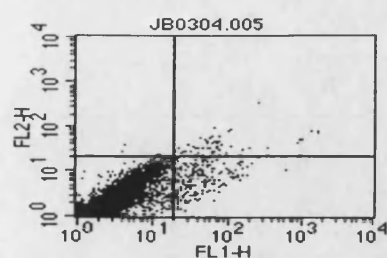
Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	7928	79.28	5.37	4.03
R3	1192	11.92	70.91	4.23
R4	162	1.62	69.78	56.98
R5	718	7.18	7.45	58.94

Figure 5.5a Sorting subpopulations of human trabecular bone-derived cells according to their expression of the Stro-1 and alkaline phosphatase cell surface antigens

Confluent first passage human trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 48 hours in DMEM containing 2% fetal calf serum and ascorbate. Cells were labelled with mouse anti-human antibodies raised against Stro-1 (IgM) and alkaline phosphatase (IgG<sub>1</sub>), then with goat anti-mouse secondary antibodies conjugated to the fluorochromes, FITC and R-PE. Cells were then separated according to the expression of Stro-1 and alkaline phosphatase by the use of fluorescence activated cell sorting (FACS).

The results are presented as dot plots: in the example above, in each graph any dot above or to the right of the central plot axis corresponds to positive labelling for Stro-1 and alkaline phosphatase (AP) respectively. Therefore the regions R2-R3 correspond to double negative, Stro-1 positive alone, positive for both antibodies and positive for AP only. Percentages of cells within each subpopulation, and the mean fluorescence intensity of the labelling of cells within each subpopulation, are recorded in tables to the right.



### secondary antibodies

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	9706	97.06	2.72	3.46
R3	8	0.08	24.42	7.24
R4	64	0.64	35.99	85.88
R5	222	2.22	6.92	41.82

### Stro-1 only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	8784	87.84	4.78	3.55
R3	917	9.17	67.42	4.51
R4	115	1.15	46.93	56.49
R5	184	1.84	9.27	44.61

### AP only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	8891	88.91	2.36	4.26
R3	2	0.02	57.00	7.67
R4	19	0.19	72.61	308.10
R5	1088	10.88	4.01	57.92

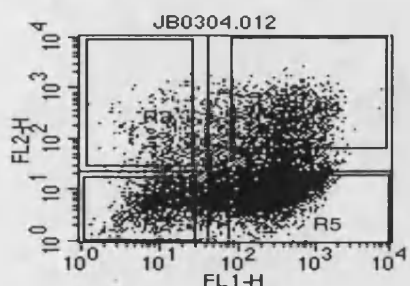
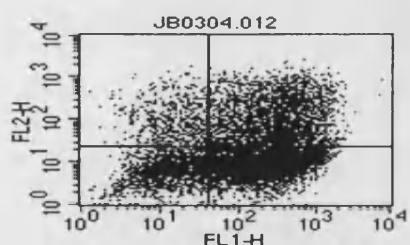
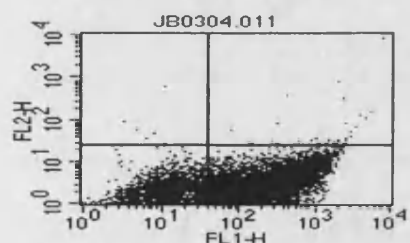
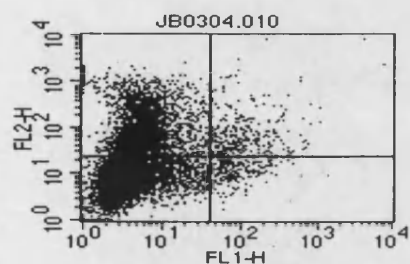
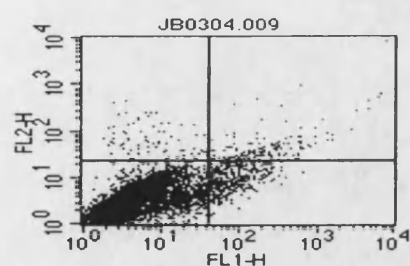
### Stro-1 and AP (dual labelling)

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	7730	77.30	4.80	4.06
R3	1167	11.67	81.77	3.75
R4	240	2.40	82.30	52.89
R5	863	8.63	6.95	60.32

Figure 5.5b The effects of calcitriol on the expression of Stro-1 and alkaline phosphatase cell surface antigens on human trabecular bone-derived cells

Confluent first passage human trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate. Cells were then exposed to 10 nM calcitriol for 24 hours before being labelled for the expression of Stro-1 and alkaline phosphatase cell surface antigens as previously described (see figure 5.5a).



### secondary antibodies

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	9320	93.20	2.90	3.97
R3	80	0.80	62.10	8.19
R4	148	1.48	48.25	232.39
R5	452	4.52	9.25	81.28

### Stro-1 only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	6080	60.80	7.73	4.01
R3	3357	33.57	90.05	5.75
R4	310	3.10	78.70	112.51
R5	253	2.53	11.46	88.31

### AP only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	2251	22.51	2.12	12.86
R3	6	0.06	70.84	6.88
R4	43	0.43	47.94	1311.12
R5	7700	77.00	3.42	257.28

### Stro-1 and AP (dual labelling)

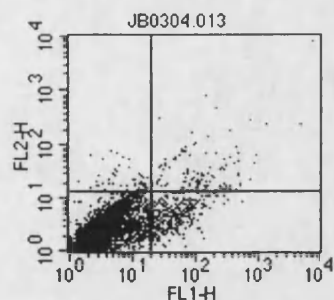
Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	1747	17.47	6.31	13.15
R3	606	6.06	95.80	15.44
R4	3107	31.07	112.00	299.84
R5	4540	45.40	8.38	222.44

Figure 5.5c The effects of dexamethasone on the expression of the Stro-1 and alkaline phosphatase cell surface antigens on human trabecular bone-derived cells

Confluent first passage human trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum, 100  $\mu$ M ascorbate-2-phosphate and 10 nM dexamethasone were cultured for 48 hours in DMEM containing 2% fetal calf serum, ascorbate and dex. Cells were then labelled for the expression of Stro-1 and AP cell surface antigens as previously described.

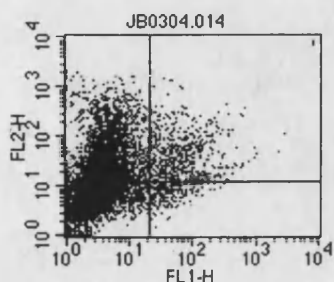




## secondary antibodies

Gated Events: 10000

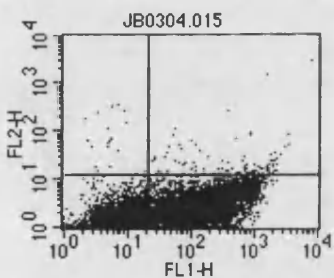
Region	Events	% Gated	X geo mean	Y geo mean
R2	9452	94.52	2.32	3.08
R3	62	0.62	20.43	8.08
R4	132	1.32	32.31	129.00
R5	354	3.54	4.37	45.78



## Stro-1 only

Gated Events: 10000

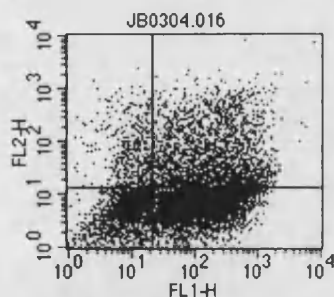
Region	Events	% Gated	X geo mean	Y geo mean
R2	7100	71.00	7.11	3.34
R3	2355	23.55	75.96	5.13
R4	284	2.84	73.40	105.71
R5	261	2.61	11.56	80.57



## AP only

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	3538	35.38	1.81	10.75
R3	19	0.19	80.01	7.38
R4	29	0.29	60.86	466.85
R5	6414	64.14	2.84	182.75



## Stro-1 and AP (dual labelling)

Gated Events: 10000

Region	Events	% Gated	X geo mean	Y geo mean
R2	2719	27.19	5.02	11.94
R3	599	5.99	86.64	14.85
R4	1471	14.71	94.17	241.14
R5	5211	52.11	7.14	185.33

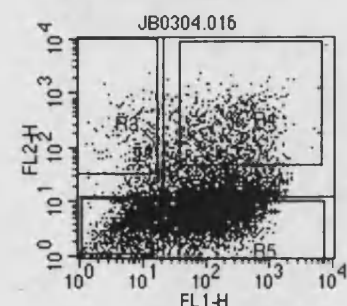


Figure 5.5d The effects of calcitriol and dexamethasone in combination on Stro-1 and alkaline phosphatase cell surface antigen expression on human trabecular bone-derived cells

Confluent first passage human trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum, 100  $\mu$ M ascorbate-2-phosphate and 10 nM dex were cultured for 24 hours in DMEM containing 2% fetal calf serum, ascorbate and dex. Cells were then exposed to 10 nM calcitriol for 24 hours prior to labelling for the expression of Stro-1 and AP cell surface antigens as previously described.

Subpopulation Factor	R2 (-/-)	R3 (+/-)	R4 (+/+)	R5 (-/+)
untreated	48 (17-79)	6.7 (1.6-12)	19 (0.2-38)	22 (6.4-38)
calcitriol	49 (20-77)	7.7 (3.7-11.7)	9.7 (1.5-18)	30 (6.8-52)
dexamethasone	14 (10-17)	3.2 (0.5-6)	23 (17-30)	58 (43-73)
dexamethasone + calcitriol	17 (6.4-27)	3.1 (0.5-6)	20 (13-26)	59 (50-68)

Table 5.4 The effects of calcitriol and dexamethasone, alone and in combination, on the number of cells expressing the Stro-1 and/ or AP cell surface antigens in cultures of human trabecular bone-derived cells

First passage trabecular bone-derived cells maintained under standard culture conditions in the absence or presence of 10 nM dexamethasone were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate, and dexamethasone as appropriate. The cells were exposed to 10 nM calcitriol for 24 hours and were then labelled for expression of Stro-1 and AP cell surface antigens as previously described and were accordingly separated using FACS analysis. The table above summarises the proportions of cells distributed between the four subpopulations following treatment with calcitriol and/ or dexamethasone as compared to untreated cells. Each value is the median from two independent experiments with the range shown in parentheses.

<u>Mean fluorescence intensity of Stro-1</u>		
<u>Subpopulation</u>	<u>R3 (+/-)</u>	<u>R4 (+/+)</u>
<u>Factor</u>		
untreated	66 (61-71)	324 (70-577)
calcitriol	67 (51-82)	60 (37-82)
dexamethasone	76 (57-96)	95 (78-112)
dexamethasone + calcitriol	58 (29-87)	66 (38-94)

<u>Mean fluorescence intensity of AP</u>		
<u>Subpopulation</u>	<u>R4 (+/+)</u>	<u>R5 (-/+)</u>
<u>Factor</u>		
untreated	483 (57-909)	84 (59-108)
calcitriol	95 (53-137)	99 (60-139)
dexamethasone	382 (300-464)	263 (222-304)
dexamethasone + calcitriol	300 (241-360)	219 (185-253)

Table 5.5 The effects of calcitriol and dexamethasone, alone and in combination, on the level of expression of Stro-1 and AP cell surface antigens on subpopulations of adult human trabecular bone-derived cells

First passage trabecular bone-derived cells maintained under standard culture conditions in the absence or presence of 10 nM dexamethasone were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate, and dexamethasone as appropriate. Cells were exposed to 10 nM calcitriol for 24 hours. The cells were then labelled for expression of Stro-1 and AP cell surface antigens as previously described and were accordingly separated using FACS analysis. The top table summarises the proportions of the mean fluorescence intensity of labelling for Stro-1, and the bottom table represents those for AP between the four subpopulations following treatment with calcitriol and/ or dexamethasone as compared to untreated cells. All values represent the median of two independent experiments with the range shown in parentheses.



### 5.6 The effects of calcitriol and dex, alone or in combination, on the expression of osteoblast-related genes in subpopulations of trabecular bone-derived cells

First passage trabecular bone-derived cells, maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M asp and in the absence or presence of 10 nM dex until confluency, were cultured for 24 hours in DMEM containing 2% fetal calf serum and 100  $\mu$ M asp and 10 nM dex as appropriate. The cells were then exposed to 10 nM calcitriol for 24 hours. RNA was harvested and analysed for the mRNA expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR (figure 5.6a) and Southern blotting (figure 5.6b). The PCRs and blots were carried out twice and average values following densitometric analysis were taken. This experiment was performed three times and in addition, the cDNA from another donor treated only with dex was supplied for analysis by Joanne Screen, B.I.R.D. The median values, with the range of values shown in parentheses were then entered into a table to facilitate comparisons between groups (table 5.6).

In the untreated cells, MSX-2 transcript expression was increased in the R5 and R2 subpopulations, when compared with R4. Treatment with calcitriol alone stimulated expression of MSX-2 mRNA in the R3 and R5 regions, whereas dex alone increased expression of this transcript in the R5 region only. Treatment with calcitriol and dex in combination resulted in an additive and supra-additive increase in the R5 and R2 subpopulations respectively, with levels of MSX-2 expression in R3 seen to be similar to those seen following treatment with calcitriol alone.

Osteocalcin mRNA expression was essentially similar between subpopulations of untreated cells. Treatment with calcitriol decreased levels in the R3 region, but stimulated those in the R5 subpopulation. Dex alone increased levels of this transcript in the R3 subpopulation. Treatment with calcitriol and dex in combination increased osteocalcin transcript levels in a supra-additive manner in the R5 and R2 subpopulations when compared with R4.

The expression of BMP-2 mRNA was seen to be increased in the R2 subpopulation and decreased in R3 when compared with R4. Calcitriol alone increased levels of this transcript in the R5 region, whereas treatment with dex alone, increased those in the R2 and R3 subpopulations. The combined treatment resulted in increased levels of this transcript in a supra-additive manner in the R5 and R2 subpopulations when compared with R4.

BMP-4 transcripts are least strongly expressed in the R2 subpopulation of untreated cells. Levels of this transcript were strongly increased in the R5 region following treatment with calcitriol, whereas dex alone increased BMP-4 mRNA expression levels in the R2 and R3 regions, when compared with R4. Treatment with calcitriol and dex in combination stimulated the expression of BMP-4 mRNA in R3 and R5, with a supra-additive increase seen in R2, when compared with R4.

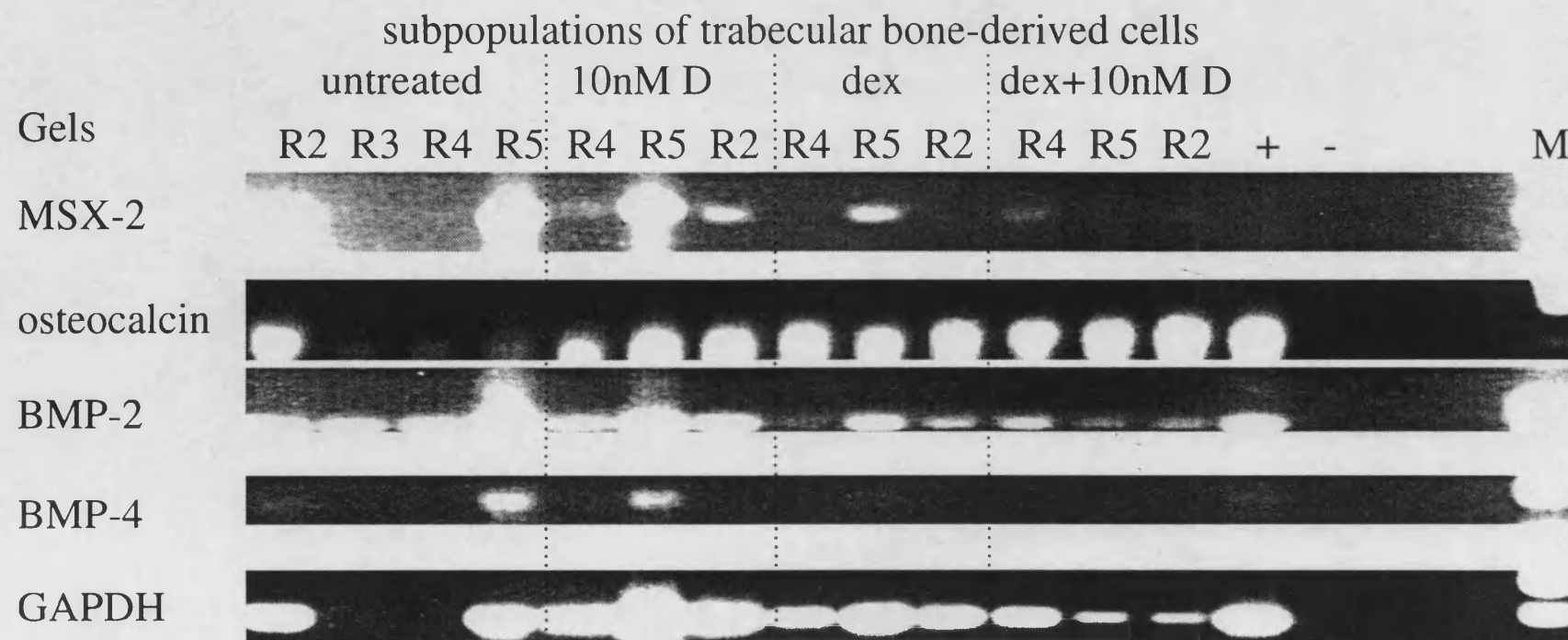


Figure 5.6a The effects of calcitriol and dexamethasone, alone or in combination, on the expression of osteoblast-related genes in subpopulations of human trabecular bone-derived cells

Confluent first passage adult human trabecular bone-derived cells maintained in DMEM supplemented with 10% fetal calf serum and 100  $\mu$ M ascorbate-2-phosphate in the absence or presence of 10 nM dexamethasone, were cultured for 24 hours in DMEM containing 2% fetal calf serum and 100  $\mu$ M ascorbate, and dexamethasone as appropriate. Cells were then exposed to 10 nM calcitriol for 24 hours prior to labelling for expression of Stro-1 and AP cell surface antigens: primary antibodies were derived from hybridoma supernatant (B4-78 hybridoma produced the mouse anti-human alkaline phosphatase IgG<sub>1</sub> which was purified by protein K column purification by Dr. C. Jefferiss, BIRD); the secondary conjugates of goat anti-mouse anti-IgM and anti-IgG were linked to FITC and R-PE. Following retrieval of the fractions containing the four subpopulations, RNA was extracted and analysed by RT-PCR and Southern blotting for MSX-2, osteocalcin, BMP-2, BMP-4 (40 cycles) and GAPDH (35 cycles) mRNA expression. PCR and Southern blotting was carried out twice for each experiment. Values obtained for direct repeats were averaged and median values, with their range shown in parentheses, were recorded in table 5.6. The gels above are representative of four independent experiments.

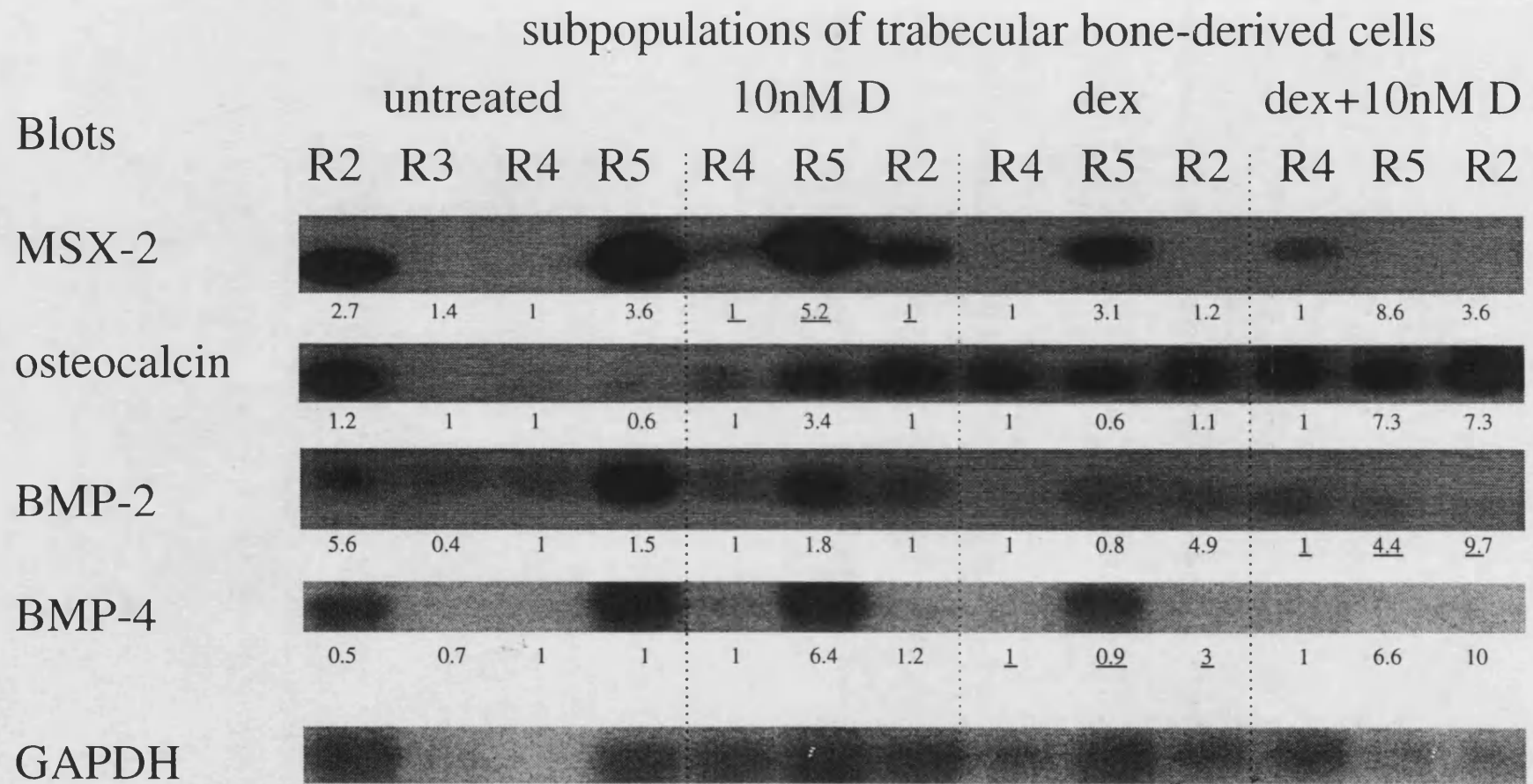


Figure 5.6b The effects of calcitriol and dexamethasone, alone or in combination, on the expression of osteoblast-related genes in subpopulations of human trabecular bone-derived cells

This figure shows Southern blots for the experiment in figure 5.6a and is representative of four independent experiments. Densitometric analysis was performed on the blots (average values shown beneath each blot). Underline indicates statistically significant H value in the Kruskal-Wallis test.

Subpopulations			R2 (-/-)	R3 (+/-)	R4 (+/+)	R5 (-/+)
Factor mRNA	10 nM calcitriol	10 nM dexamethasone				
MSX-2	-	-	2.7 (0.3-18)*	1.4 (1-3.3)*	1*	3.6 (0.6-4.7)*
		+	1.2 (1.1-1.5)*	1.3 (1.3-4.6)*	1†	3.1 (2.4-3.7)~
	+	-	1 (0.6-2.5)*	3.6 (1.5-5.8)~	1~	5.2 (3.5-7)~
		+	3.6 (2.3-4.9)~	2.9≈	1~	8.6 (2.4-15)~
osteocalcin	-	-	1.2 (0.5-1.9)~	1 (1-3.3)*	1*	0.6 (0.4-0.7)~
		+	1.1 (1.1-2.8)*	2.4 (1.3-3.5)~	1†	0.6 (0.2-2.4)*
	+	-	1 (0.2-2)*	0.4 (0.2-0.6)~	1~	3.4 (1.6-5.3)~
		+	7.3 (5.8-8.9)~	1.6≈	1~	7.3 (3.3-11)~
BMP-2	-	-	5.6 (0.2-11)~	0.4 (0.2-1)†	1*	1.5 (1.3-1.8)~
		+	4.9 (0.4-11.7)†	1.9 (0.2-40)*	1†	0.8 (0.2-3.6)†
	+	-	1 (0.6-1.7)*	0.9 (0.6-1.3)~	1~	1.8 (1.5-2.2)~
		+	9.7 (8.7-11)~	1.7≈	1~	4.4 (2-6.7)~
BMP-4	-	-	0.5 (0.2-17)*	0.7 (0.3-2.5)†	1*	1 (0.5-46)*
		+	3 (1.5-18)†	4 (1.5-25)*	1†	0.9 (0.4-16)†
	+	-	1.2 (1-1.4)~	1.4≈	1~	6.4 (5.1-7.7)~
		+	10 (9.3-11)~	6.4≈	1~	6.6 (6.4-6.9)~

Table 5.6 The effects of calcitriol and dexamethasone, alone or in combination, on the expression of osteoblast-related genes in subpopulations of adult human trabecular bone-derived cells

Confluent cells maintained under standard culture conditions and in the absence or presence of dexamethasone were cultured for 24 hours in DMEM containing 2% fetal calf serum and ascorbate, and dexamethasone as appropriate. The cells were treated with 10 nM calcitriol for 24 hours and then were harvested and labelled for the cell surface expression of Stro-1 and AP antigens. Cells were accordingly separated using FACS analysis. RNA was extracted from each subpopulation and examined for the mRNA expression of MSX-2, osteocalcin, BMP-2, BMP-4 and GAPDH by RT-PCR and Southern blotting. These latter two processes were performed twice for each independent experiment. Densitometry was performed and the values obtained for repeats of PCRs and Southern blots were averaged. The table above represents the median values obtained from up to four independent experiments. ≈Value obtained from one experiment. ~Average value from two experiments; \* average value from three experiments; † average value from four experiments, with the range of values shown in parentheses.

Statistical analysis to compare gene expression patterns between the four subpopulations of adult human trabecular bone-derived cells was performed and can be seen in null hypothesis 24, Appendix 3.

### Discussion

Long term culture of cells in the presence of dexamethasone had marked effects on cell morphology: both bone marrow- and trabecular bone-derived cells exhibited a smooth, elongated, fibroblast-like appearance when cultured under normal conditions, but in the presence of dexamethasone, morphology was truncated, and cells appeared more polygonal and therefore more osteoblastic in appearance. This is consistent with the observations by Cheng et al (1994) and with the finding that dexamethasone stimulates osteoblastic differentiation, although this effect is dependent upon the stage of cell maturation (reviewed by Delany et al, 1994). The change in morphology may be mediated by the reduction in the attachment of the cells to their extracellular matrix (Gronowicz and McCarthy, 1995).

Treatment of bone marrow-derived cells with dexamethasone resulted in a reduced proportion of AP negative cells (cells in the R2 and R3 fractions) and increased numbers in the AP positive regions (R4 and R5). Untreated trabecular bone-derived cells display a similar pattern. The effects of dexamethasone on both human trabecular bone- and bone marrow-derived cells suggest that culture in the presence of dexamethasone stimulates the progression of these cells from R2 to R5, and therefore drives the terminal differentiation of these cells at all stages of maturation. Within the R2 fraction also, there may be represented a proportion of cells driven through to terminal differentiation. This is supported by the finding that expression of bone sialoprotein, a molecule expressed only by mineralising cells (osteoblasts, odontoblasts and cementoblasts), is upregulated by dexamethasone (Sodek et al, 1995) as mediated by the presence of a glucocorticoid response element (GRE) in its promoter (Ogata et al, 1995). The reduced numbers of cells in the R2 fraction, however, imply that there are fewer osteogenic precursors. This supports the conclusion that treatment with glucocorticoids reduces the proliferation of osteogenic precursors (Doherty et al, 1995). By conjecture, fewer precursors would result in a reduced pool of osteogenic cells in the future, suggesting a mechanism whereby dexamethasone might cause a reduction in bone mineral density.

When compared with control, calcitriol modestly reduced the number of cells in the R3 fraction of bone marrow-derived cells, and increased that in the R4 and R5 subpopulations: calcitriol modestly decreased the proportion of cells in the R4 subpopulation and stimulated that in the R5 fraction of trabecular bone-derived cells. This suggests that calcitriol promotes the maturation of cells already progressing through differentiation, but effects only minor changes in the precursor populations. Treatment with calcitriol in combination with dex was seen to have no effect on the distribution of cells between the subpopulations when compared with dex alone, suggesting that acute treatment (less than 48 hours) with calcitriol is insufficient to neutralise the effects of dex on the number of cells expressing these surface antigens.

To investigate further the effects of these agents on the expression of Stro-1 and AP antigens, changes in the levels of antigen expression on the cells were examined by

the use of mean fluorescence intensity measurements. In bone marrow-derived cells, dexamethasone decreases the expression of Stro-1 in the R3 and R4 subpopulations but greatly increased that of AP in the R4 and R5 fractions. Therefore, taking these results together, dexamethasone stimulates the number of cells expressing AP in the R4 and R5 subpopulations, and increases the expression level of AP on these cells. The reduction in the level of Stro-1 expression on the R3 and R4 fractions combined with increases in AP again suggests that the number of precursors is decreased (R2 and R3 fractions). This supports the observation earlier that dexamethasone enhances the differentiation of osteogenic cells while reducing the pool of precursors, and relates to the observations made in fig. 5.1, where dexamethasone treatment altered the morphology of the fibroblast-like cultured cells to a more osteoblastic one.

In bone marrow-derived cells, calcitriol alone has little effect on the expression levels of the Stro-1 antigen, but stimulates levels of AP expression, thereby promoting the specialisation of cells already progressing through differentiation. This suggests again that the primary actions of calcitriol are on maturing cells, and not on precursors. Calcitriol antagonises the stimulation of AP expression by dexamethasone to levels still greater than control: in the R4 and R5 subpopulations, cell numbers are unaffected whereas AP expression levels are decreased, when compared to the effects of dexamethasone alone. Expression levels of Stro-1 antigen are increased in the R4 subpopulation. Taking these results together, treatment with calcitriol modulates the differentiative effects of dex. Therefore, it would appear that in bone marrow-derived cells, co-treatment with calcitriol modulates the expression levels of the AP antigen but does not affect the number of cells expressing it when compared to the actions of dex alone. When compared to the effects of dex, co-treatment with calcitriol does not affect the level of expression of Stro-1 antigen in the R3 subpopulation but modulates the decrease elicited by dex in the R4 region, and does not greatly affect the number of cells expressing it in any of the fractions. This supports the idea that calcitriol has little effect on precursors.

In trabecular bone-derived cells, dex, alone or in combination with calcitriol, decreases expression levels of Stro-1 and AP in R4 relative to untreated cells, concomitant with an increase in AP expression in R5 region. This, the increase in numbers in the R5 fraction, and the decrease in the number of cells in the R2 and R3 regions following treatment with dex, alone or in combination with calcitriol, suggests that dex promotes the terminal differentiation of maturing trabecular bone-derived cells, and strongly reduces the proliferation of their precursors. Levels of Stro-1 and AP antigen expression are reduced in the R4 subpopulation following treatment with calcitriol, but AP antigen expression levels are modestly increased in R5. This and the stimulation of cell numbers in the R5 suggests that calcitriol also acts as a maturation-enhancing agent, primarily affecting cells already differentiating.

Therefore, in trabecular bone- and bone marrow-derived cells, treatment with dex, alone or in combination, stimulates the differentiation of the precursor cells and reduces

their numbers, and increases the number of terminally differentiated osteoblastic cells (R5), when compared to untreated cells. By conjecture, in the short-term, the number of terminally differentiated cells is increased, so initially producing more bone, but with a reduced number of precursors, there would be an eventual decrease in the number of cells capable of producing bone. Acute treatment with calcitriol does not ameliorate the long-term effects of dexamethasone on the numbers of differentiated cells in cultures of trabecular bone- and bone marrow-derived cells.

As differences were seen between trabecular bone- and bone marrow-derived cells and between the effects of dex and calcitriol on the numbers of cells expressing Stro-1/AP cell surface antigens and/ or the levels of antigen expressed on each cell, thus supporting the suggestion that the actions of these agents may be maturation-dependent, the separation of cells according to their cell surface expression of the Stro-1 and alkaline phosphatase antigens was shown to be appropriate.

RT-PCR was employed to examine the pattern of gene expression in the subpopulations of cells from bone marrow and trabecular bone. To reiterate, this technique is unsuitable for absolute quantitation of the magnitude of change in gene expression levels. However, within the limitations of RT-PCR, and emphasising any changes in direction only in the expression of osteoblast-related genes, consistent trends were observed. The expression of the genes of interest was seen to be restricted to certain subpopulations, but the patterns were different in bone marrow- and trabecular bone-derived cells. In bone marrow-derived cells, the pattern of expression in the untreated control cultures suggests that transcripts for BMP-4 and MSX-2 are maximally expressed earlier in the differentiation cascade (AP negative, R3) than BMP-2 (Stro-1 negative, R5) and osteocalcin (Stro-1 negative, R4/R5). Treatment with calcitriol stimulates expression of BMP-2, BMP-4 and osteocalcin earlier in the cascade (R2), thereby promoting the early differentiation of cells. Interestingly, the expression of MSX-2 was increased in R2 but the maximal increase was in R3: the dramatic increase in MSX-2 in this region may downregulate the expression of osteocalcin in the R3 fraction.

The potent stimulation of MSX-2, osteocalcin and BMP-2 in the AP negative subpopulations (R2 and R3) by dexamethasone confirms the conclusion that dexamethasone enhances the early specialisation of these bone marrow-derived cells. One known effect of glucocorticoids is to suppress the replication of preosteoblasts (Doherty et al, 1995): this is confirmed by the FACS data where numbers of cells present in the R2 and R3 fractions are reduced by long-term culture in dexamethasone. Therefore the galvanisation of differentiation in a reduced number of cells, accompanied by a reduction in the number of osteoblastic precursors, can be related to the effects of dexamethasone *in vivo*, where decreased bone formation (Cosman et al, 1994) occurs as a result of the production of fewer mature osteoblasts (Doherty et al, 1995). The increased expression of BMP-4 in the Stro-1 negative, AP positive subpopulation (R5) when compared with control, where BMP-4 transcript expression is downregulated coincident with



downregulation in Stro-1 antigen expression, suggests that the continued expression of this gene may contribute to the increased number of cells in the R5 region or to the differentiation of this subpopulation following treatment with dexamethasone.

Treatment with calcitriol and dexamethasone in combination increases BMP-2 mRNA expression in the AP negative fractions (R2 and R3) and suppresses expression in the R5 fraction, when compared to the effects of dexamethasone alone. There is some degree of antagonism between the two agents as the maximal increase following treatment with both agents is lower than that seen following treatment with dexamethasone alone. The magnitude of maximal BMP-4 transcript expression, also in the R3 fraction, indicates that the agents were acting independently of each other as an additive increase was seen. Expression of MSX-2 and osteocalcin mRNA is maximally stimulated in the R2 fraction: MSX-2 expression in the Stro-1 negative fractions (R5 and R2) was increased in a supra-additive manner; osteocalcin transcript levels in the R5 region were also greater than in those seen following treatment with either agent alone, but the maximal increase (in the R2 region) indicated some antagonism between the agents as levels were lower than following treatment with dexamethasone alone.

Taken together, these data suggest that calcitriol alone acts as a maturation-enhancing agent, promoting the specialisation of cells by further increasing the expression of osteoblast-related genes in early subpopulations. Dexamethasone acts at all levels and promotes differentiation and maturation, but reduces the number of osteogenic precursors. Dexamethasone has a more dominant effect on the expression of osteoblast-related genes in bone marrow-derived cells than calcitriol when the two agents are added in combination, as calcitriol can only partly antagonise the effects of dexamethasone on the expression of these genes. The interactions between these two agents produced additive or supra-additive effects on the maximal expression of BMP-4 and MSX-2 transcripts respectively, which occurred in subpopulations where the cell numbers were decreased, suggesting that the expression of these genes is involved in the increased differentiation of bone marrow-derived cells.

Interestingly, when variation in the levels of transcript expression in the control subpopulations was taken into account, the expression pattern in bone marrow-derived cells in the R2 fraction following treatment with calcitriol was seen to closely resemble that seen in MG-63 cells after exposure to the agent. Expression of all four genes was increased, with maximal increase observed in BMP-2, then osteocalcin, MSX-2 and BMP-4. This supports the idea that MG-63 cells can be used as an adequate model of osteoblastic differentiation. A further step would be to label these osteosarcoma cells for the expression of Stro-1 and alkaline phosphatase and examine how these vary following treatment with calcitriol and/ or dexamethasone, and also how the expression of the genes of interest change within the subpopulations.

While calcitriol and dexamethasone, alone or in combination, increase the level of expression of osteoblast-related transcripts in both trabecular bone- and bone marrow-

derived cells, the gene expression pattern in untreated trabecular bone-derived cells is different in detail from that seen in untreated bone marrow-derived cells. In the control, maximal levels of BMP-2 and osteocalcin are seen in the R2 region (respectively AP positive R5 and R4 fractions in bone marrow-derived cells), those of BMP-4 in the R4/R5 regions and those of MSX-2 in the Stro-1 negative R5/R2 fractions (both expressed maximally in the Stro-1 positive R3 region for bone marrow-derived cells). This pattern of gene expression (BMP-4-MSX-2-BMP-2-osteocalcin) is similar to the expression pattern outlined for human bone-derived cells previously (in order, BMP-2-BMP-4-MSX-2-osteocalcin, see results chapters 1 and 2). Treatment with calcitriol induces maximal levels of expression of transcripts for all four genes in the R5 fraction but the magnitude is decreased for BMP-2, when compared with control. Therefore in these cells, calcitriol maximally induces osteoblast-related gene expression in cells positive for alkaline phosphatase cell surface expression only, that is, in terminally differentiated cells.

Dexamethasone treatment induces a maximal increase in MSX-2 in R5 (R3 in bone marrow-derived cells), and BMP-4 and osteocalcin in the Stro-1 positive R3 region (Stro-1 negative R5 and R2 subpopulations in bone marrow-derived cells, respectively). Osteocalcin transcript expression is suppressed in the R5 subpopulation by treatment with dexamethasone, which is consistent with findings that dexamethasone inhibits the expression of osteocalcin in osteoblast cultures (reviewed by Delany et al, 1994). Maximal increase in BMP-2 expression was in the R2 region (Stro-1 positive R3 region in bone marrow-derived cells), and a decrease was seen in the R5 subpopulation: respectively, the proportion of cells was decreased and increased when compared with control (see table 5.4). Therefore, following treatment with dexamethasone, expression of BMP-2 mRNA is increased in reduced numbers of undifferentiated cells, and reduced in the increased numbers of terminally differentiated cells. This suggests that expression of BMP-2 transcripts is related to the proliferative capability of the cell.

Calcitriol and dexamethasone in combination produce additive effects on the magnitude of maximal expression of transcripts for MSX-2, BMP-2 and BMP-4: expression levels for osteocalcin transcripts are increased in a supra-additive manner. Levels of transcript expression of all four genes are increased in the Stro-1 negative (R5 and R2) subpopulations (AP negative R2 and R3 fractions in bone marrow-derived cells) when compared to the action of dexamethasone alone, suggesting that the action of calcitriol and dexamethasone in concert promotes the terminal differentiation of these cells. That this stimulation of differentiation occurs in the R2 cell population, where cell numbers are decreased when compared to untreated cells or cells treated with calcitriol alone, supports the idea that this subpopulation can contain cells which are fully differentiated.

Of the four subpopulations of cells separated according to their expression of Stro-1 and alkaline phosphatase, the one which is most ambiguous is the double negative

population (R2): neither Stro-1 nor alkaline phosphatase is expressed on the cell surface of these cells. As the methodology stands, it is impossible to discover if this fraction represents a population which is early and undifferentiated, fully differentiated or a mixture of both. Ideally, the development and application of an antibody which could distinguish between primitive precursors and terminally differentiated osteoblasts could at least partly answer this. However, in trabecular bone- or bone marrow-derived cells, the reduction in precursor proliferation by dexamethasone decreased the number of cells in this subpopulation, and the increase in osteocalcin transcript expression in the R2 region following treatment with calcitriol and dexamethasone in combination indicates that the R2 subpopulation contains terminally differentiated cells. Therefore, it is likely that, following treatment with calcitriol and dexamethasone, the R2 subpopulation contains a mixture of both undifferentiated and terminally differentiated cells. Extrapolating these findings to an *in vivo* situation, a substantial decrease in the numbers of precursors would result in fewer mature osteoblasts when the precursors differentiate, which in turn would cause a net imbalance in bone remodelling. By conjecture and as previously discussed, acute treatment with calcitriol would not rescue the detrimental effects to bone formation of constant exposure to dexamethasone.

Taken together, these data support the conclusion that calcitriol and dexamethasone are both osteogenic differentiation agents, increasing the expression of osteoblast-related genes including *MSX-2*, but in contrast to the finding for bone marrow-derived cells, calcitriol has the more dominant effect in trabecular bone-derived cells. Also, while the agents have broadly similar effects on cell numbers and modulation of gene expression in trabecular bone- and bone marrow-derived cells, the cell subpopulations in which the effects of these agents occur differ. This implies that, while the effects of the agents are maximal in specific subpopulations and therefore in specific maturation stages, the maturation stages at which these effects take place are different between trabecular bone- and bone marrow-derived cells.

One explanation for differences in the patterns of gene expression in cells derived from bone marrow and trabecular bone, untreated or following treatment with dexamethasone and/ or calcitriol, could be that the bone marrow-derived cells are progressing through differentiation for the first time and as a consequence, no inhibitions on the expression of genes later in the cascade have been placed. Trabecular bone contained cells at different maturation stages, therefore the culturing of trabecular bone-derived cells may have resulted in de-differentiation, where when first plated, cells already involved in the cascade of differentiation are returned to a less mature state by the presence of proliferative signals from the medium or, as cell density is known to have an effect on gene expression, intercellular communication may be involved.

While separation of cells using FACS was seen to provide some indication that the expression of osteoblast-related genes may be cell maturation-dependent, some variation in the response of cells to treatment with exogenous factors was still apparent. Therefore,

the other factors indicated previously (age, gender, site of extraction of bone, etc.) must play a role in the regulation of expression of these genes. Indeed, it was shown recently that increased androgen serum levels and greater expression of the androgen receptor at specific skeletal sites in males contributes to gender-related differences in the morphology of the skeleton (Kasperk et al, 1997). Appendix 2 contains a donor list, including age, gender, extraction site and whether the patient contributed trabecular bone, marrow or both. Six patients donated marrow (four female, two male; all but one above the age of 50 years; all but one contributed rib); twelve patients donated trabecular bone (five female, seven male; all but three above the age of 60 years; at least four sites of extraction). In the absence of sufficient tissue samples, these parameters could not be tested here, therefore an obvious next step in an attempt to discern the factors affecting osteoblast-related gene expression in osteoblastic cells would be to test the effects of the exogenous growth factors in age- and gender-matched donors following extraction of samples from the same region of bone.

This results chapter shows that there are differences in the effects of calcitriol and dexamethasone on trabecular bone- and bone marrow-derived cells. Overall, both agents are potent differentiative factors and regulate the expression of osteoblast-related genes, including *MSX-2*. The regulation by these agents of the expression of these genes, and therefore of differentiation, was seen to be maturation-specific and was related to the source of cells (bone marrow or trabecular bone). Dexamethasone reduced the number of precursors and stimulated the progressive differentiation of all cells at different stages of maturation. Co-treatment with calcitriol resulted in increased numbers of terminally differentiated cells but did not affect the anti-proliferative effects of dexamethasone on early, immature cells. A net decrease in the number of mature osteoblasts, and therefore of bone formation, as a result of reduced proliferation of precursors following dexamethasone treatment would not be substantially altered following subsequent acute treatment with calcitriol, despite an initial increase in the number of terminally differentiated cells. Thus, by conjecture, dexamethasone-induced osteoporosis may not be reversed by acute treatment with calcitriol.

## **Chapter 6**

### **DISCUSSION AND FUTURE WORK**

Classically, the study of homeobox-containing genes has centred on their role in the patterning and differentiation of cells in the development of the embryo (reviewed by Gehring, 1994). The importance of such genes in morphogenetic pathways, for example in the development and outgrowth of the limb, and at other sites of epithelial-mesenchymal interactions, such as craniofacial development and tooth morphogenesis, has been underlined in a comprehensive body of research in rodent and avian embryos. The discovery that the homeobox-containing gene *MSX-2* is expressed at these sites has implicated its homeodomain-containing protein product in the regulation of these processes. Also shown is that *MSX-2* expression is induced by the bone morphogenetic proteins in a paracrine fashion: signalling by epithelially-produced BMP-4 induces expression of BMP-2, BMP-4 and *MSX-2* in underlying mesenchyme and is a key feature of morphogenesis.

In this project, it has been consistently shown that *MSX-2* mRNA expression in adult human bone-derived cells is induced by BMPs -2 and -4. The finding that the same regulation of *MSX-2* by BMPs in the differentiation of the mesenchymally-derived osteoblastic cell lineage occurs in adult tissue as in embryonic tissue development has important implications for the understanding of osteogenesis and the maintenance of bone as an organised tissue in the adult, and by inference, in the comprehension of the breakdown of such processes in the development of osteoporosis. It was suggested that the tightly restricted expression of *MSX-2* is critical in the switch between proliferation and differentiation, and that it acts as a mediator of BMP-induced osteogenesis. Its function is not conclusively clear in these studies, but it has been shown to act as a negative regulator of chondrogenesis (Mina et al, 1996) and, as BMPs act as adipogenic antagonists (Gimble et al, 1995), it is possible that *MSX-2* mediates this negative differentiation signal also. It is therefore possible that *MSX-2* expression and subsequent downregulation acts primarily as a negative regulator of chondrogenesis/ adipogenesis, leading to the formation of bone by default, or appropriate *MSX-2* expression could actively stimulate osteogenesis. This has obvious implications for the development of age-related bone disease such as osteoporosis, where inappropriate expression of *MSX-2* could conceivably result in a strong drive away from osteogenesis and towards adipogenesis/ chondrogenesis. Examination of the function of *MSX-2* in bone cells is clearly important, therefore disabling the functions of *MSX-2* by development of an *MSX-2* knock-out mouse or introduction of antisense oligonucleotides to cells in culture would be a vital step in the understanding of the role of this protein in bone cell biology.

Regulation by the pleiotropic steroid hormone metabolite calcitriol appears to be conserved between cells derived from osteosarcoma and from normal adult tissue. This regulation was shown to involve indirect mechanisms. Exploration of the precise mechanisms involved requires knowledge of the promoter sequence, therefore cloning of

sequence further upstream from the 1.2 kb sequence already elucidated is necessary to confirm that the effects of calcitriol on the expression of MSX-2 are indirect. It is possible that some or all of the E box sequences in the 1.2 kb promoter region are functional and therefore may play a critical role in the regulation of the expression of this gene. In order to test this, firstly it would be of interest to ensure that binding occurs at these sites. Therefore use of oligonucleotide sequences identical to these motifs in an electromobility shift assay (EMSA) would determine if cell proteins bind to any of these sequences. A next step would be to fuse the promoter sequence to a reporter gene, such as beta-galactosidase, luciferase or chloramphenicol acetyl transferase, to determine if any of these sequences are functional. Disruption of these sequences by deletion analysis or in vitro mutagenesis could examine if a loss of function occurs.

Once it is clear that E box proteins bind to the MSX-2 promoter and are functional, activating transcription, investigations into the role of Id (inhibitor of differentiation) genes can take place. Competition using Id proteins in an EMSA to examine a proposed loss of binding at the E box protein binding sites would be a first step. Co-transfection of a construct containing coding sequence for Id protein fused to an inducible promoter (EG) and the construct containing the MSX-2 promoter linked to a reporter gene already described could show that transcription of the reporter gene is reduced or ablated when the Id construct is activated.

Use of the RT-PCR technique revealed consistent trends in the effects of the parameters tested upon the expression of osteoblast-related genes. However, as stated in the Discussion sections of each Results chapter, this technique is not appropriate for the absolute quantitation of changes in magnitude of gene expression. Therefore, it would be of interest to analyse the RNA from these experiments with another more quantitative method, such as dot-blot hybridisation.

Use of FACS analysis was shown to be appropriate: the results showed exactly what the experiments were designed to show, namely that the expression of osteoblast-related genes is dependent upon the stage of maturation of the cell, that the effects of agents on the expression of these genes is also stage-specific, even to the extent of being opposite at different stages, and that differences between donors may reflect the varying proportions of cells at different stages of maturation/ differentiation. The proposed model for the cascade of osteoblastic gene expression was partially upheld: in untreated bone marrow-derived cells, BMP-4 is expressed earliest, then MSX-2 and osteocalcin. Further work where cells are separated by FACS, then recultured and treated with calcitriol and dexamethasone may elucidate the position of BMP-2 in the cascade. It would be useful also to monitor the expression of Stro-1 and AP over time in these sorted and replated subpopulations. Of great interest also would be to understand where in the progression of

cells from progenitor to mature osteoblast that BMPs have their greatest effects. This could be examined by treating these sorted and recultured cells with BMP-2 or BMP-4.

The discovery that the calcitropic agents calcitriol and dexamethasone have maturation-specific effects on the expression of osteoblast-related genes warrants further investigation. Separation of cells by FACS followed by treatment with these factors could elucidate their effects, as the fractions obtained in the course of this research were not 100% pure. The repercussions of the effects of these agents in clinical terms suggests mechanisms whereby drug-induced osteopenia and osteoporosis might occur. The results shown suggest that dexamethasone strongly suppresses the replication of osteoblast precursors, thus reducing the pool of osteoprogenitors which can differentiate into mature bone-forming cells. It would therefore be of great interest to examine agents which could be shown to counteract the detrimental effects of dexamethasone on bone formation. Preliminary data following treatment of bone cell cultures with fibroblast growth factor-2 (FGF-2) indicates that this agent is a potent stimulator of osteogenic proliferation. Indeed, use of FGFs in stimulating bone formation has already been shown (Dunstan et al, 1993). Studies on the interactions between these factors could be of clinical importance in the prevention or treatment of osteoporosis.

Cell: cell and cell: matrix communication was seen modulate the effects of the agents studied on the expression of osteoblast-related genes. This may be mediated by the levels of adhesion molecules and the prevalence of gap junctional communication between cells. It would be appropriate to examine the levels of the expression of these molecules at different cell densities, in the presence and absence of extracellular matrix, and to investigate how these levels might be modulated by the agents studied.

The interactions between osteogenic factors in this project indicate a complex regulation of osteoblast-related gene expression. This would seem sensible when considered in terms of the *in vivo* situation, where several factors are present at or near the bone-forming surface and osteoblasts at any given time. Local concentrations, and therefore effects, of these factors will vary according to rates of synthesis, sequestration to the matrix and degradation, and importantly upon the expression levels of their receptors (nuclear vitamin D receptor and cell surface serine/threonine kinase receptors). An obvious next step would be to examine the levels of expression of these receptor molecules as cells proliferate and differentiate, and how their ligands might modulate them.

The regulation of osteogenesis by the CBFA1 gene was comprehensively examined recently (reviewed by Rodan and Harada, 1997). To recap, CBFA1 is expressed in all prospective endochondral bone sites and by intramembranous craniofacial bone, as



derived from the branchial arches (Komori et al, 1997). In situ hybridisation studies indicate that CBFA1 is expressed in osteoblastic and chondroblastic bipotential cells during mesenchymal condensation but continues to be expressed only in cells of the osteoblast lineage (Ducy et al, 1997). The expression patterns of CBFA1 and MSX-2 are therefore coincident. It would be of great interest to examine the expression of both genes simultaneously in adult human bone-derived cells, and to investigate the effects of the BMPs on the expression of CBFA1. Interestingly, in the 1.2 kb MSX-2 promoter region, there are no consensus binding sites for CBFA1 (see figure 1.5 and 4.10), therefore it would be of great interest to observe if the expression of these two genes is inter-related.

The preceding chapters indicate that osteoblastic differentiation is influenced in a complex manner in adult human bone-derived cells by osteoinductive agents, including calcitriol, bone morphogenetic proteins and dexamethasone. A more extensive investigation into the expression of other genes in the osteoblast differentiation pathway, such as type I collagen, osteonectin, osteopontin and bone sialoprotein, and how they are affected by these osteogenic agents, would be of great interest to further characterise intermediate stages in the progression of precursors to osteoblast maturity, and thereby to increase understanding of how these cells lose function over time, and why with age, osteoblasts become relatively less abundant.

## **APPENDIX 1**

### **Stock solutions**

**Tissue culture**

DMEM (Dulbecco's modified Eagle's medium):

100 ml	10x DMEM (+NEAA, -glutamine, -pyruvate, -bicarbonate)
20 mM	Hepes buffer
0.09%	sodium bicarbonate
1 mM	sodium pyruvate
2 mM	L-glutamine
25 IU-25 µg	penicillin/streptomycin

This was made up to 1 litre with Milli-Q water and adjusted to pH 7.2 with sodium hydroxide before filter sterilisation. It was then aliquoted into 500 ml autoclaved bottles and stored at 4°C. (All tissue culture stocks were supplied by Gibco BRL).

Phosphate buffered saline (PBS):

10 PBS tablets were dissolved in 1 litre of Milli-Q water and autoclaved. It was allowed to cool to room temperature before use.

Collagenase IV:

This was made up in sterile water or serum-free medium and stored in 1 ml aliquots at -20°C. It was used at 25 U/ ml final concentration.

Deoxyribonuclease I (DNase I):

This was made up in 150 mM sodium chloride to 2000 units/ ml. It was used at 20 units/ ml final concentration.

Transforming growth factor-beta-1 (TGF-beta-1):

This was constituted in 4 mM hydrochloric acid plus 1 mg/ ml bovine serum albumin and stored in 500 µl aliquots at -20°C.

Bone morphogenetic proteins 2, 4 and 6 (BMP-2, BMP-4 and BMP-6):

These were made up in DMEM and stored in 500 µl aliquots (BMP-2 at 100 µg/ ml, BMP-4 and BMP-6 at 25 µg/ ml) at -20°C.

Bone morphogenetic protein 7 (BMP-7):

This was made up in DMEM and stored in sealed evacuated tubes in 40 µl aliquots of 100 µg/ ml at -20°C.

1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (calcitriol):

This was made up in absolute ethanol and stored in sealed evacuated tubes under gaseous nitrogen in 250 µl aliquots of 3.44x 10<sup>4</sup> M at -20°C.

Basic fibroblastic growth factor (FGF-2, NBS Biologicals):

This was made up in PBS and stored in 10 µl aliquots of 50 µg/ml at -20°C.

Ascorbate-2-phosphate (asp):

This was supplied as a desiccant and stored at -20°C in a box containing desiccant. It was made up in DMEM to a stock strength of 10 mM and filter-sterilised before being stored at -20°C as 5 ml aliquots. It was used at a final concentration of 100 µM.

Dexamethasone (dex):

This was made up in serum-free DMEM to a stock concentration of  $5 \times 10^{-5}$  M and filter-sterilised before being stored at -20°C as 1 ml aliquots. It was used at a final concentration of  $10^{-8}$  M.

### FACS

Hanks balanced salt solution (HBSS) x10 stock:

54 mM	potassium chloride
4 mM	potassium hydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )
2 M	sodium chloride
4 mM	disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ )

This was made to 1 litre with distilled water and stored. Prior to use, it was diluted in distilled water and autoclaved at 15 lb/in<sup>2</sup> for 30 minutes.

Blocking buffer:

10%	AB serum
5%	fetal calf serum (FCS)
1%	bovine serum albumin (BSA)
made up in HBSS	

An appropriate quantity of BSA was measured and dissolved in HBSS. To this were added the two types of serum and the volume adjusted with HBSS. If the blocking buffer was required to be sterile, the BSA dissolved in HBSS was filter-sterilised prior to the addition of the sterile sera. Blocking buffer was made fresh at the start of each experiment.

HHF:

10 mM	Hepes
5%	FCS
made up in HBSS	

This was made fresh at the start of each experiment and used to increase volumes of cell suspensions prior to centrifugation and to collect cell fractions following FACS analysis.

**RT-PCR**

Random hexamer primers [Pd(N)<sub>6</sub>]:

1 ml of sterile water was added to 50 U Pd(N)<sub>6</sub> and gently pipetted up and down to suspend. This was stored at -20°C.

PCR primers:

These were produced by Pharmacia Biotech and required dilution to 20-30 pmoles/ µl before use in the PCR. Concentrated stocks and ready-to-use diluted primers were stored in aliquots at -20°C.

**Gel electrophoresis**

DEPC-treated water:

1 ml of diethylpyrocarbonate (DEPC) was added to 1 litre of Milli-Q water in a fume cupboard. This was covered and left mixing on a magnetic stirrer overnight. The following day, the treated water was autoclaved, then allowed to cool to room temperature before use.

10x TAE gel running buffer:

400 mM	Tris base
50 mM	sodium acetate
10 mM	EDTA

This was diluted in distilled water and brought to pH 8.1 with glacial acetic acid so that when diluted to working strength (1x) the running pH was 7.9.

5x TBA gel running buffer:

445 mM	Tris base
445 mM	boric acid
10 mM	EDTA

No pH adjustment was necessary for this concentrated buffer stock once made up in distilled water.

50x MOPS, pH 7.0 (RNA gel running buffer):

1 M	MOPS
250 mM	sodium acetate
50 mM	EDTA, pH 7.0

This was adjusted to pH 7.0 with sodium hydroxide and autoclaved. It turned a characteristic golden colour and was thereafter stored in the dark.

**Blotting reagents**

20x SSC:

175.3 g	sodium chloride
88.2 g	sodium citrate

800 ml distilled water was added then the solution was adjusted to pH 7.0 with hydrochloric acid. It was made up to 1 litre and filter-sterilised or autoclaved.

1M sodium phosphate, pH 7.2:

89 g	sodium phosphate, dibasic
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This was adjusted to pH 7.2 by addition of 3-4 ml concentrated orthophosphoric acid and autoclaved.

0.4 M sodium hydroxide:

8 g sodium hydroxide pellets were added to 500 ml distilled water and mixed on a magnetic stirrer until dissolved.

50x Denhardt's solution:

5 g	Ficoll
5 g	polyvinylpyrrolidone (PVP)
5 g	bovine serum albumin

This was made up to 500 ml with Milli-Q water and filter-sterilised before storage at -20°C.

Denatured salmon sperm DNA:

This was supplied desiccated and was reconstituted by addition of water. It was made up to 8-10 mg/ ml final stock concentration and sheared by sonication. Following phenol:chloroform extraction, the DNA was placed in aliquots of 1 ml and stored at -20°C.

Bromophenol blue dye:

0.25%	bromophenol blue
15%	Ficoll 40

This was made up in DEPC-treated water for RNA gel electrophoresis analysis.

Church's buffer:

0.5 M	sodium phosphate, pH 7.2 (see above)
7%	sodium dodecyl sulphate (SDS)
1 mM	EDTA, pH .0

This was made up to 1 litre with DEPC-treated water and stored at room temperature. To ensure even distribution of SDS, all the buffer was heated to hybridisation temperature and mixed before an aliquot was removed for the hybridisation reaction.

Wash buffer:

40 mM	sodium phosphate, pH 7.2
1%	SDS

This was made up in distilled water and stored at room temperature.

### **General solutions**

10x TE, pH 8.0:

100 mM	Tris base, pH 8.0
10 mM	EDTA, pH 8.0

This was made up to 100 ml with distilled water and autoclaved.

### **Bacterial culture**

Ampicillin:

This was made up as a 25 mg/ ml stock in Milli-Q water and sterilised by filtration before being stored at -20°C in 5 ml aliquots.

Bacterial growth medium:

10 g	Bacto-tryptone (Unipath Ltd.)
5 g	Bacto-yeast extract (Unipath Ltd.)
10 g	sodium chloride

This was made up to 1 litre with distilled water and autoclaved at 120°C for 20 minutes before use.

## **APPENDIX 2**

### **Donor information**



Donor number	Type of bone donated	Age	Gender	Site of extraction
486	marrow	84	female	femoral head
537	marrow	73	female	rib
301	trabecular	69	female	femoral head
323	trabecular	89	female	femoral head
502	trabecular	66	female	iliac crest
409	marrow and trabecular	39	female	rib
523	marrow and trabecular	61	female	rib
518	marrow	54	male	rib
286	trabecular	21	male	fibula fracture
310	trabecular	32	male	fibula
326	trabecular	64	male	not recorded
331	trabecular	63	male	knee
372	trabecular	74	male	rib
484	trabecular	64	male	knee
524	marrow and trabecular	72	male	rib

The table above lists the characteristics of the donors who contributed tissue to this research. Of the donations, 12 were of trabecular bone and 6 were of bone marrow. To be noted is the age range (21- 89 years) and the variety of sites of extraction, which include rib, femoral head, knee and fibula.

## **APPENDIX 3**

### **Statistical analysis**

Null hypothesis 1:

Calcitriol has no effect on the level of osteoblast-related gene expression in MG-63 cells

Calcitriol (M)	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
0	1	1	1	1	1	1	1	1	1	1	1	1
10 <sup>-7</sup> M	4			6			3			4		
10 <sup>-8</sup> M	6	6	6	14	2	3	28	1	1	6	3	4
10 <sup>-9</sup> M	0	1	2	1	3	3	1	1	2	1	1	2
critical H value												
at 5% significance	6.333			6.333			6.333			6.333		
calculated												
H values	4.333			<b>6.333</b>			<b>6.333</b>			4.333		

The calculated H value is only significant if it is equal to or greater than the critical H value. Therefore, only the effects of calcitriol on BMP-2 and osteocalcin expression are statistically significant and the null hypothesis of calcitriol having no effect on the expression of these genes in MG-63 cells must be rejected. Calcitriol appears to have no effect on the expression of the mRNA of the other genes tested, therefore as the null hypothesis is upheld for these genes, the second part of the Kruskal-Wallis test, for Q values, was not performed upon them.

## Q values

For this set of experiments, where the number of samples equals 4, the critical Q value is 2.639. If the calculated value is greater than or equal to the critical value, a significant difference between the two samples is indicated. Throughout the Q value sections in Appendix 3, numbers in bold type indicate an increase at 5% significance when the vertical axis is compared to the horizontal, and those underlined show a decrease.

## Osteocalcin expression

0				
10 <sup>-7</sup>				1.768
10 <sup>-8</sup>		0.289	2.122	
10 <sup>-9</sup>	1.061	0.707	1.500	
10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	0	

These data indicate no significant differences in osteocalcin gene expression following treatment with any dose of calcitriol when compared to untreated control.

## BMP-2 expression

0				
10 <sup>-7</sup>			1.768	
10 <sup>-8</sup>		0.289	2.122	
10 <sup>-9</sup>	1.061	0.707	1.500	
	10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	0

Therefore there is no significant increase in BMP-2 expression following treatment with any dose of calcitriol.

Therefore, while calcitriol produces an overall significant stimulatory effect on the expression of osteocalcin and BMP-2, this is not due to differences in effect between specific doses.

Null hypothesis 2: BMP-2 does not affect osteoblastic gene expression in MG-63 cells

BMP-2 (ng/ml)	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
0	1	1	1	1	1	1	1	1	1	1	1	1
1	1.5			2.6			5.8			3.6		
50	1.5	2.5	36	1.0	1.3	1.6	1.5	1.7	3.6	0	0	0.3
250	2.3			1.3			0			0.2		
critical H value												
at 5% significance	6.333			6.333			6.333			6.333		
calculated												
H values	5.500			4.333			5.833			5.889		

BMP-2 has no statistically significant effect on the expression of any of the genes tested, therefore the null hypothesis that BMP-2 has no effect on osteoblast-related gene expression in MG-63 cells must be upheld.

Null hypothesis 3:

BMP-7 does not affect the transcript levels of osteoblast-related genes in MG-63 cells

BMP-7 (ng/ml)	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
0	1	1	1	1	1	1	1	1	1	1	1	1
1	10.8			4.8			6.4			9.0		
10	4.3			24			15			24		
50	2.8	3.4	14	1.8	2.9	13	1.5	1.7	3.9	0.9	1.0	8.6
250	10			6.3			4.5			4.4		
critical H value												
at 5% significance	7.111			7.111			7.111			7.111		

calculated

H values	5.867	6.578	<b>7.467</b>	4.911
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BMP-7 treatment was seen to have a significant effect only on the expression of BMP-2 transcripts. Therefore the null hypothesis that BMP-7 has no effect on BMP-2 mRNA expression in MG-63 cells must be rejected.

Q values

As 5 samples are being compared, the critical Q value is 2.807.

BMP-2 expression

0

1				1.898
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10		0.258	2.214	
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50		1.265	0.949	1.342
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250	0.633	0.516	0.258	1.581
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250	50	10	1	0
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There were no significant differences in BMP-2 mRNA expression following treatment with BMP-7.

Therefore, treatment with BMP-7 has an overall significant stimulatory effect on BMP-2 mRNA expression which cannot be related to specific doses.

#### Null hypothesis 4:

TGF-beta has no effect on the expression of osteoblast-related genes in MG-63 cells

TGF-beta (ng/ml)	relative MSX-2 expression	relative osteocalcin expression	relative BMP-2 expression	relative BMP-4 expression
0	1 1 1	1 1 1	1 1 1	1 1 1
0.05	3 8 67	1 2 5	0 1 6	1 1 7
0.25	7	4	8	6
1.25	3	4	2	5
critical H value				
at 5% significance	6.333	6.333	6.333	6.333
calculated				
H values	5.222	5.222	3.222	3.638

No significant effects on osteoblast-related gene expression were seen following treatment of MG-63 cells with TGF-beta. Therefore the null hypothesis is upheld.

Statistical analyses of the effects of BMP-4 and BMP-6 on osteoblast-related gene expression in MG-63 cells were not done as insufficient independent repeats of the experiments were performed.

The following section examines the expression of osteoblast-related genes in MG-63 cells treated with a combination of the above agents at the following doses: 10nM calcitriol (D), 50 ng/ml BMP-2 (B2) or BMP-7 (B7) and 0.05 ng/ml TGF-beta (Tb).

#### Null hypothesis 5:

Interactions between calcitriol and increasing doses of BMP-2 have no effect on osteoblast-related gene expression in MG-63 cells

Agent	relative MSX-2 expression	relative osteocalcin expression	relative BMP-2 expression	relative BMP-4 expression
10 nM D	2 6 6	2 3 14	1 1 28	3 4 6
1 ng/ ml BMP-2 + D	1.5	2	2	1
50 ng/ ml BMP-2 + D	3 3 160	2 3 4	0.5 1 2	0.5 1 3
250 ng/ ml BMP-2 + D	0	12	3	2
critical H values	6.333	6.333	6.333	6.333
calculated H values	4.111	0.833	2.556	5.222

No significant difference between the treatment groups was seen, therefore the null hypothesis is upheld.

#### Null hypothesis 6:

Interactions between calcitriol and increasing doses of BMP-7 have no effect on osteoblast-related gene expression in MG-63 cells

Agent	relative MSX-2 expression	relative osteocalcin expression	relative BMP-2 expression	relative BMP-4 expression
10 nM D	2 6 6	2 3 14	1 1 28	3 4 6
1 ng/ ml BMP-7 + D	10	11	9	22
10 ng/ ml BMP-7 + D	9	31	8	24
50 ng/ ml BMP-7 + D	7 19 327	4 7 21	4 5 10	3 11 13
250 ng/ ml BMP-7 + D	9	9	3	12
critical H values	7.111	7.111	7.111	7.111
calculated H values	5.867	3.377	1.778	5.688

No significant difference between the treatment groups was seen, therefore the null hypothesis is upheld.

#### Null hypothesis 7:

Interactions between calcitriol and increasing doses of TGF-beta have no effect on osteoblast-related gene expression in MG-63 cells

Agent	relative MSX-2 expression	relative osteocalcin expression	relative BMP-2 expression	relative BMP-4 expression
10 nM D	2 6 6	2 3 14	1 1 28	3 4 6
0.05 ng/ ml TGF + D	5 5 119	2 5 9	1 3 4	0 2 4
0.25 ng/ ml TGF + D	4	3	2	5
1.25 ng/ ml TGF + D	4	4	2	8
critical H values	6.333	6.333	6.333	6.333
calculated H values	2.111	0.222	0.111	4.111

No significant difference between the treatment groups was seen, therefore the null hypothesis is upheld.

#### Null hypothesis 8:

Calcitriol and BMP-2, alone or in combination, have no effect on osteoblast-related gene expression in MG-63 cells

Agent	relative MSX-2 expression	relative osteocalcin expression	relative BMP-2 expression	relative BMP-4 expression
untreated	1 1 1	1 1 1	1 1 1	1 1 1
calcitriol	0 1 2	1 3 3	1 2 2	0.5 1 2
BMP-2	0.5 3 11	1 1 2	2 2 4	0 0.1 0.2
calcitriol + BMP-2	1 1 2	2 2 3	1 1 2	0 0.1 0.4
critical H values	7.000	7.000	7.000	7.000
calculated H values	2.077	6.885	5.654	<b>8.436</b>

The only significant difference was in BMP-4 transcript expression between the treatment groups, therefore the null hypothesis that calcitriol and BMP-2, alone or in combination, do not affect BMP-4 mRNA expression must be rejected.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

## BMP-4 expression

untreated

calcitriol			0.340
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BMP-2		2.266	1.926
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calcitriol + BMP-2	0.112	2.150	1.810
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	BMP-2	calcitriol	untreated
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Calcitriol and BMP-2, alone or in combination, have no significant effect on BMP-4 gene expression in MG-63 cells.

Therefore, the result of treatment with calcitriol and BMP-2, alone or in combination, have an overall inhibitory effect on BMP-4 expression which cannot be related to the effects of either agent alone or in combination.

Null hypothesis 9:

Calcitriol and BMP-7, alone or in combination, have no effect on osteoblast-related gene expression in MG-63 cells

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
calcitriol	0	1	2	1	3	3	1	2	2	1	1	2
BMP-7	3	3	14	2	3	13	2	2	4	1	1	9
calcitriol + BMP-7	1	5	8	2	3	6	1	3	7	0.5	2	7
critical H values	7.000			7.000			7.000			7.000		
calculated H values	<b>12.462</b>			<b>7.192</b>			4.089			0.423		

The only significant differences were in MSX-2 and osteocalcin transcript expression between the treatment groups, therefore the null hypothesis that calcitriol and BMP-7, alone or in combination, do not affect MSX-2 and osteocalcin mRNA expression must be rejected.

## Q values

As 4 samples are being compared, the critical Q value is 2.639.

## MSX-2 expression

untreated

calcitriol		0.567
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BMP-7	2.154	1.586
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calcitriol + BMP-7	0	2.154	1.586
	BMP-7	calcitriol	untreated

Calcitriol and BMP-7, alone or in combination, have no significant effect on MSX-2 gene expression in MG-63 cells.

Osteocalcin expression

untreated

calcitriol			1.471
BMP-7		0.849	2.320
calcitriol + BMP-7	0	0.849	2.320
	BMP-7	calcitriol	untreated

Calcitriol and BMP-7, alone or in combination, have no significant effect on osteocalcin gene expression in MG-63 cells.

Therefore, the stimulatory effects of calcitriol and BMP-7, alone or in combination, on osteocalcin expression were shown to be significant overall but could not be related to specific interactions. Similarly, the inhibitory effects of calcitriol on MSX-2 expression relieved by treatment with BMP-7 were not shown to be specifically significant, despite the overall significance of the effects of the agents on MSX-2 expression, as indicated by the calculated H value.

#### Null hypothesis 10:

Calcitriol and TGF-beta, alone or in combination, have no effect on osteoblast-related gene expression in MG-63 cells

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
calcitriol	0	1	2	1	3	3	1	2	2	0.5	1	2
TGF-beta	3	7	67	1	2	5	0	1	6	1	1	7
calcitriol + TGF-beta	0	2	3	1	3	4	0	3	4	0	0.4	1
critical H values	7.000			7.000			7.000			7.000		
calculated H values	6.859			6.320			1.500			6.269		

There was no significant difference in the transcript expression of any of the genes tested following treatment with calcitriol and TGF-beta, alone or in combination, therefore the null hypothesis is upheld.

#### Null hypothesis 11:

Members of the TGF-beta superfamily, alone or in combination, have no effect on osteoblast-related gene expression in MG-63 cells

1) Compare against BMP-2 + BMP-7

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-2	0.5	3	11	1	1	2	2	2	4	0	0.1	0.2
BMP-7	3	3	14	2	3	13	2	2	4	1	1	9
TGF-beta	3	8	67	1	2	5	0	1	6	1	1	7
BMP-2+ BMP-7	6			3			2			7		
BMP-2, BMP-7 + TGF-beta	12			5			0			0		
critical H values	11.07			11.07			11.07			11.07		
calculated H values	7.095			8.848			5.962			9.419		

There was no significant difference in the transcript expression of any of the genes tested following treatment with calcitriol and TGF-beta, alone or in combination, therefore the null hypothesis is upheld.

2) Compare against BMP-2 + TGF-beta

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-2	0.5	3	11	1	1	2	2	2	4	0	0.1	0.2
BMP-7	3	3	14	2	3	13	2	2	4	1	1	9
TGF-beta	3	8	67	1	2	5	0	1	6	1	1	7
BMP-2+ TGF-beta	16			2			1			3		
BMP-2, BMP-7 + TGF-beta	12			5			0			0		
critical H values	11.07			11.07			11.07			11.07		
calculated H values	8.009			8.847			7.800			9.190		

There was no significant difference in the transcript expression of any of the genes tested following treatment with calcitriol and TGF-beta, alone or in combination, therefore the null hypothesis is upheld.

3) Compare against BMP-7 + TGF-beta

Agent	relative			relative			relative			relative		
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	MSX-2 expression			osteocalcin expression			BMP-2 expression			BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-2	0.5	3	11	1	1	2	2	2	4	0	0.1	0.2
BMP-7	3	3	14	2	3	13	2	2	4	1	1	9
TGF-beta	3	8	67	1	2	5	0	1	6	1	1	7
BMP-7+ TGF-beta	28			4			1			10		
BMP-2, BMP-7 + TGF-beta	12			5			0			0		
critical H values	11.07			11.07			11.07			11.07		
calculated H values	8.009			8.809			8.562			10.962		

There was no significant difference in the transcript expression of any of the genes tested following treatment with calcitriol and TGF-beta, alone or in combination, therefore the null hypothesis is upheld.

#### Null hypothesis 12:

Overexpression of MSX-2 has no effect on the induction of MSX-2 expression in osteoblast-like cells by calcitriol

Cells	untreated	MG-63	MC3T3-E1	ROS 17/ 2.8	R2/ 8	R2/ 11
MSX-2 expression	1	0.1	0.8	1	2.1	0.4
	1	0.4	1.3	1	2.5	0.7
	1			1.2		
	1			2.1		
critical H value	11.07					
calculated H value	8.267					

There was no significant difference in the transcript expression of any of the genes tested following treatment with calcitriol and TGF-beta, alone or in combination, therefore the null hypothesis is upheld.

In the following section, the effects of the osteogenic factors on the expression of osteoblast-related genes in adult human trabecular bone-derived cells are examined.

#### Null hypothesis 13:

Calcitriol has no effect on the level of osteoblast-related gene expression in adult human trabecular bone-derived cells

Calcitriol (M)	relative	relative	relative	relative
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	MSX-2 expression					osteocalcin expression					BMP-2 expression					BMP-4 expression				
0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10 <sup>-7</sup> M	1.2					1.3					0.9					1.2				
10 <sup>-8</sup> M	1	1.1	1.9	3.1	3.7	1.1	1.2	6.4	18.6	18.7	0.5	0.7	0.9	3.1	3.5	0.8	0.8	1.8	2.4	3.1
10 <sup>-9</sup> M	1.2					1					0.5					0.6				
10 <sup>-10</sup> M	0.8					0.8					0.3					0.9				
10 <sup>-11</sup> M	0.1					0.4					0.2					0.5				
Critical																				
H values					11.07					11.07					11.07					11.07
Calculated																				
H values					9.240					10.960					7.131					6.097

No significant effect of calcitriol on the expression of any of the genes of interest was seen, therefore the null hypothesis is upheld.

#### Null hypothesis 14:

BMP-2 has no effect on the level of osteoblast-related gene expression in adult human trabecular bone-derived cells

BMP-2 (ng/ ml)	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
0	1	1	1	1	1	1	1	1	1	1	1	1
5	6.4			3.4			6.0			12.4		
10	22.7			7.4			14.1			14.3		
20	42.7			5.1			15.3			19.9		
50	11.5			4.3			12.2			11.4		
100	0.8	2.2		12.8	15.0		3.5	4.6		6.9	8.0	
Critical												
H values			7.467			7.467			7.467			7.467
Calculated												
H values			6.667			<b>7.667</b>			<b>7.667</b>			<b>7.667</b>

BMP-2 was seen to have significant effects on the expression of osteocalcin, BMP-2 and BMP-4 mRNA, therefore the null hypothesis that BMP-2 does not affect the expression of these genes must be rejected. BMP-2 was not shown to have a significant effect on the expression of MSX-2 transcripts, therefore the null hypothesis is upheld for the expression of this gene following treatment with the agent.

#### Q values

The critical Q value for 6 samples is 2.936.

## Osteocalcin expression

0					
5					0.633
10				0.775	1.581
20			0.258	0.516	1.265
50		0.258	0.516	0.258	0.949
100	1.044	0.745	0.447	1.342	2.600
	50	20	10	5	0

The effect of BMP-2 on osteocalcin expression in adult human trabecular bone-derived cells cannot therefore be related to the dose of BMP-2.

## BMP-2 expression

0					
5					1.265
10				0.516	1.898
20			0.258	0.775	2.214
50		0.516	0.258	0.258	1.581
100	0.745	1.342	1.044	0.447	1.000
	50	20	10	5	0

There is no significant effect of BMP-2 on BMP-2 expression in adult human trabecular bone-derived cells.

## BMP-4 expression

0					
5					1.581
10				0.258	1.898
20			0.258	0.516	2.214
50		0.775	0.516	0.258	1.265
100	0.447	1.342	1.044	0.745	1.000
	50	20	10	5	0

There is no significant effect of BMP-2 on BMP-4 expression in adult human trabecular bone-derived cells.

Therefore, the overall significant stimulation in osteocalcin, BMP-2 and BMP-4 mRNA expression elicited by BMP-2 treatment was not shown to be statistically related to the dose of the agent when individual treatment groups were compared.

Null hypothesis 15:

BMP-7 has no effect on the level of osteoblast-related gene expression in adult human trabecular bone-derived cells

BMP-7 (ng/ ml)	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
0	1	1	1	1	1	1	1	1	1	1	1	1
10	0.8			3.0			1.6			1		
20	8.7			4.8			7.1			1.9		
50	16.0			1.3			11.1			4.8		
100	0.5	1.4	5.3	4.1	16.5	20.9	1.6	2.5	4.1	0.4	0.7	2.0
Critical												
H values		7.111			7.111			7.111			7.111	
Calculated												
H values		4.978			<b>7.111</b>			<b>7.244</b>			3.511	

BMP-7 was seen to have significant effects on the expression of osteocalcin and BMP-2 mRNA, therefore the null hypothesis that BMP-7 does not affect the expression of these genes must be rejected. The null hypothesis is upheld for the expression of MSX-2 and BMP-4 as following treatment with BMP-7 no statistically significant effect was seen.

## Q values

The critical Q value for 5 samples is 2.807.

## Osteocalcin expression

0				
10				0.949
20			0.516	1.581
50		0.775	0.258	0.633
100	1.094	0.200	0.796	2.268
	50	20	10	0

There is no significant effect of BMP-7 on osteocalcin expression in adult human trabecular bone-derived cells.

## BMP-2 expression

0			
10			0.791
20		0.904	1.898
50	0.258	1.162	2.214

100	0.945	0.647	0.397	1.532
	50	20	10	0

There is no significant effect of BMP-7 on BMP-2 expression in adult human trabecular bone-derived cells.

Therefore, the overall significant stimulation in osteocalcin and BMP-2 expression elicited by treatment with BMP-7 was not seen to be specifically related to dose of the agent when individual treatment groups were compared.

#### Null hypothesis 16:

Calcitriol and BMP-2, alone or in combination, have no effect on osteoblast-related gene expression in adult human trabecular bone-derived cells

Agent	relative MSX-2 expression					relative osteocalcin expression					relative BMP-2 expression					relative BMP-4 expression				
untreated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
calcitriol	1	1.8	1.9	3.1	3.7	1	1.2	6.4	18.6	18.7	0.5	0.9	1.2	3.1	3.5	0.8	1.4	1.8	2.4	3.1
BMP-2	0.8	2.2				12.8	15.0				3.5	4.6				6.9	8.0			
calcitriol + BMP-2	3.1	4.3				31.5	34.1				4.1	4.9				6.5	7.3			
critical H values	6.777					6.777					6.777					6.777				
calculated H values	<b>7.004</b>					<b>9.326</b>					<b>7.867</b>					<b>9.343</b>				

There were significant differences in the transcript expression of all of the genes tested following treatment with calcitriol and BMP-2, alone or in combination, therefore the null hypothesis is rejected with regard to the expression of all four genes.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### MSX-2 expression

untreated

calcitriol 1.776

BMP-2 1.057 0.286

calcitriol + BMP-2 1.733 1.014 2.357

BMP-2 calcitriol untreated

Calcitriol and BMP-2, alone or in combination, have no significant effect on MSX-2 gene expression in adult human trabecular bone-derived cells.

#### Osteocalcin expression

untreated

calcitriol			1.814
BMP-2		0.343	1.714
calcitriol + BMP-2	0.956	1.486	<b>2.857</b>
	BMP-2	calcitriol	untreated

Calcitriol and BMP-2 in combination have a significant effect on osteocalcin gene expression in adult human trabecular bone-derived cells when compared to untreated cells. No other significant effect was seen.

BMP-2 expression			
untreated			
calcitriol			0.416
BMP-2		1.614	1.929
calcitriol + BMP-2	0.299	1.971	2.286
	BMP-2	calcitriol	untreated

Calcitriol and BMP-2, alone or in combination, have no significant effect on BMP-2 gene expression in adult human trabecular bone-derived cells.

BMP-4 expression			
untreated			
calcitriol			1.134
BMP-2		1.714	2.571
calcitriol + BMP-2	0.239	1.429	2.286
	BMP-2	calcitriol	untreated

Calcitriol and BMP-2, alone or in combination, have no significant effect on BMP-4 gene expression in adult human trabecular bone-derived cells.

Therefore, the overall significant stimulation seen in the expression levels of all genes tested was largely not seen when comparisons between individual treatment groups were made. The only specific significant stimulation was seen in osteocalcin expression following treatment with calcitriol and BMP-2 in combination when compared to untreated control cells.

#### Null hypothesis 17:

Calcitriol and BMP-7, alone or in combination, have no effect on osteoblast-related gene expression in adult human trabecular bone-derived cells

Agent	relative MSX-2 expression					relative osteocalcin expression					relative BMP-2 expression					relative BMP-4 expression				
untreated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
calcitriol	1	1.8	1.9	3.1	3.7	1	1.2	6.4	18.6	18.7	0.5	0.9	1.2	3.1	3.5	0.8	1.4	1.8	2.4	3.1
BMP-7	0.5	1.4	5.3			4.1	16.5	20.9			1.6	2.5	4.1			0.4	0.7	2.0		



calcitriol				
+ BMP-7	0.9 1.8	24.8 26.2	2.8 3.6	0.1 0.7
critical H values	7.815	7.815	7.815	7.815
calculated H values	4.135	<b>10.167</b>	6.102	6.700

There were significant differences only in the transcript expression of osteocalcin following treatment with calcitriol and BMP-7, alone or in combination, therefore with this exception, the null hypothesis is upheld.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### Osteocalcin expression

untreated

calcitriol			1.768
BMP-7		0.560	2.091
calcitriol + BMP-7	1.022	1.603	<b>2.940</b>
	BMP-7	calcitriol	untreated

Calcitriol and BMP-7 in combination have a significant effect on osteocalcin gene expression in adult human trabecular bone-derived cells when compared to untreated cells. No other significant effect was seen.

Similar to the observations following treatment of adult human trabecular bone-derived cells with calcitriol and BMP-2 in combination, the overall significant stimulation in expression levels of osteocalcin was largely not seen when individual treatment groups were compared, except for the augmentation in expression observed when the cells were treated with calcitriol and BMP-7 in combination as compared to untreated cells.

#### Null hypothesis 18:

Calcitriol and TGF-beta, alone or in combination, have no effect on osteoblast-related gene expression in adult human trabecular bone-derived cells

Agent	relative MSX-2 expression					relative osteocalcin expression					relative BMP-2 expression					relative BMP-4 expression				
untreated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
calcitriol	1	1.8	1.9	3.1	3.7	1	1.2	6.4	18.6	18.7	0.5	0.9	1.2	3.1	3.5	0.8	1.4	1.8	2.4	3.1
TGF-beta	2.5	3.1					10.5	12.9			2.4	3.8				4.5	5.3			
calcitriol																				
+TGF-beta	3.0	3.6					25.8	29.2			2.7	3.7				1.5	1.9			
critical H values						6.777					6.777					6.777				

There were significant differences in the transcript expression of MSX-2, osteocalcin and BMP-4 following treatment with calcitriol and TGF-beta, alone or in combination, therefore with the exception of BMP-2 expression, which was not enhanced following treatment with calcitriol and TGF-beta alone or in combination, the null hypothesis must be rejected.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### MSX-2 expression

untreated

calcitriol 2.003

TGF-beta 0.414 1.929

calcitriol + TGF-beta 0.299 0.771 2.286

TGF-beta calcitriol untreated

Calcitriol and TGF-beta, alone or in combination, have no significant effect on MSX-2 gene expression in adult human trabecular bone-derived cells.

#### Osteocalcin expression

untreated

calcitriol 1.814

TGF-beta 0.343 1.714

calcitriol + TGF-beta 0.956 1.486 **2.857**

TGF-beta calcitriol untreated

Calcitriol and TGF-beta in combination have a significant effect on osteocalcin gene expression in adult human trabecular bone-derived cells when compared to untreated control. No other significant effect was seen.

#### BMP-4 expression

untreated

calcitriol 1.512

TGF-beta 1.571 **2.714**

calcitriol + TGF-beta 1.076 0.286 1.429

TGF-beta calcitriol untreated

TGF-beta alone has a significant effect on BMP-4 gene expression in adult human trabecular bone-derived cells when compared to untreated cells. No other treatment, alone or in combination, was seen to be significant.

Interestingly, this experiment yielded overall significant stimulation in the expression of all the genes tested, with the exception of BMP-2. Following comparison of the individual treatment groups, osteocalcin and BMP-4 expression was stimulated by treatment with calcitriol and TGF-beta in combination and TGF-beta alone when compared to untreated control, respectively.

#### Null hypothesis 19:

Calcitriol and FGF-2, alone or in combination, have no effect on osteoblast-related gene expression in adult human trabecular bone-derived cells

Agent	relative MSX-2 expression					relative osteocalcin expression					relative BMP-2 expression					relative BMP-4 expression				
untreated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
calcitriol	1	1.8	1.9	3.1	3.7	1	1.2	6.4	18.6	18.7	0.5	0.9	1.2	3.1	3.5	0.8	1.4	1.8	2.4	3.1
FGF-2	2.8					14.2					3.0					1.1				
calcitriol +FGF-2	4.6					0.5					2.3					1.2				

critical H values	6.077	6.077	6.077	6.077
calculated H values	<b>7.023</b>	<b>7.023</b>	2.323	4.292

There were significant differences in the transcript expression of MSX-2 and osteocalcin following treatment with calcitriol and FGF-2, alone or in combination, therefore for these two genes only, the null hypothesis must be rejected.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### MSX-2 expression

untreated			
calcitriol			1.930
FGF-2		0.278	1.646
calcitriol + FGF-2	0.588	1.038	2.152
	FGF-2	calcitriol	untreated

Calcitriol and FGF-2, alone or in combination, have no significant effect on MSX-2 gene expression in adult human trabecular bone-derived cells.

#### Osteocalcin expression

untreated			
calcitriol			1.930
FGF-2		0.278	1.392
calcitriol + FGF-2	1.765	2.000	0.866

Therefore, the overall significant stimulation in MSX-2 and osteocalcin transcript levels was not related to specific effects of individual treatment groups.

Members of the TGF-beta superfamily, alone or in combination, have no effect on osteoblast-related gene expression in MG-63 cells

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-2	0.8	2.2		12.8	15.0		3.5	4.6		6.9	8.0	
BMP-7	0.5	1.4	5.3	4.1	16.5	20.9	1.6	2.5	4.1	0.4	0.7	2.0
BMP-2 +BMP-7	1.1	1.2		16.3	19.3		1.6	2.0		5.9	7.1	
critical H values	6.527			6.527			6.527			6.527		
calculated H values	1.181			6.472			7.277			6.818		

## Q values

### BMP-2 expression

BMP-2			2.533
BMP-7		0.785	1.954
BMP-2 + BMP-7	0.572	1.238	1.176
	BMP-7	BMP-2	untreated

### BMP-4 expression

BMP-2 1.809

BMP-7		2.171	0.405
BMP-2 + BMP-7	1.809	0.330	1.447
	BMP-7	BMP-2	untreated

BMP-2 and BMP-7, alone or in combination, have no significant effect on BMP-4 gene expression in adult human trabecular bone-derived cells.

Therefore, the overall significant stimulation in BMP-2 and BMP-4 transcript levels was not seen following comparison of individual treatment groups.

## 2) Compare BMP-2 and TGF-beta

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-2	0.8	2.2		12.8	15.0		3.5	4.6		6.9	8.0	
TGF-beta	2.5	3.1		10.5	12.9		2.4	3.8		4.5	5.3	
BMP-2 +TGF-beta	1.4	5.3		12.4	14.8		2.7	3.0		4.1	4.9	
critical H values	6.333			6.333			6.333			6.333		
calculated H values	4.933			5.933			<b>6.333</b>			<b>7.133</b>		

There were significant differences in the transcript expression of BMP-2 and BMP-4 following treatment with BMP-2 and TGF-beta, alone or in combination, therefore for these two genes only, the null hypothesis must be rejected.

## Q values

As 4 samples are being compared, the critical Q value is 2.639.

## BMP-2 expression

untreated			
BMP-2			2.400
TGF-beta		0.730	1.600
BMP-2 + TGF-beta	0.183	0.913	1.400
	TGF-beta	BMP-2	untreated

BMP-2 and TGF-beta, alone or in combination, have no significant effect on BMP-2 gene expression in adult human trabecular bone-derived cells.

## BMP-4 expression

untreated		
BMP-2		2.600
TGF-beta	0.548	1.600

BMP-2 + TGF-beta	0.365	1.278	1.200
	TGF-beta	BMP-2	untreated

BMP-2 and TGF-beta, alone or in combination, have no significant effect on BMP-4 gene expression in adult human trabecular bone-derived cells.

As for the previous experiment, the overall significant stimulation in BMP-2 and BMP-4 mRNA expression levels was not seen following comparison of individual treatment groups.

### 3) Compare BMP-7 and TGF-beta

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-7	0.5	1.4	5.3	4.1	16.5	20.9	1.6	2.5	4.1	0.4	0.7	2.0
TGF-beta	2.5	3.1		10.5	12.9		2.4	3.8		4.5	5.3	
BMP-7 +TGF-beta	5.9	9.5		21.2	23.8		4.1	4.7		0.5	1.6	
critical H values	6.527			6.527			6.527			6.527		
calculated H values	5.981			7.727			7.727			4.527		

There were significant differences in the transcript expression of osteocalcin and BMP-2 following treatment with BMP-7 and TGF-beta, alone or in combination, therefore for these two genes only, the null hypothesis is rejected.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### Osteocalcin expression

untreated

BMP-7			1.752
TGF-beta		0.300	1.266
BMP-7 + TGF-beta	1.321	1.147	2.713
	TGF-beta	BMP-7	untreated

BMP-7 and TGF-beta in combination have a significant stimulatory effect on osteocalcin gene expression in adult human trabecular bone-derived cells when compared to untreated cells. No other significant effect was seen.

#### BMP-2 expression

untreated

BMP-7		1.687
TGF-beta	0.062	1.447

BMP-7 + TGF-beta	1.073	1.114	2.623
	TGF-beta	BMP-7	untreated

BMP-7 and TGF-beta, alone or in combination, have no significant effect on BMP-2 gene expression in adult human trabecular bone-derived cells.

The overall stimulation in BMP-2 transcript levels was not seen following comparisons between the individual treatment groups. However, following treatment with both agents in combination, osteocalcin mRNA levels were significantly increased when compared to those seen in the untreated control.

#### Null hypothesis 21:

Members of the TGF-beta superfamily, alone or in combination with FGF-2, have no effect on osteoblast-related gene expression in MG-63 cells

#### 1) Compare BMP-2 and FGF-2

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-2	0.8	2.2		12.8	15.0		3.5	4.6		6.9	8.0	
FGF-2	2.8			14.2			3.0			1.1		
BMP-2 +FGF-2	2.3			0.1			2.5			1.2		
critical H values	5.833			5.833			5.833			5.833		
calculated H values	4.083			5.833			6.083			6.083		

There were significant differences in the transcript expression of osteocalcin, BMP-2 and BMP-4 following treatment with BMP-2 and FGF-2, alone or in combination, therefore with the exception of MSX-2 expression, which was seen to be unaffected by treatment, the null hypothesis is rejected.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

## Osteocalcin expression

untreated

BMP-2			1.278
FGF-2		0	1.650
BMP-2 + FGF-2	2.000	1.732	0.913
	FGF-2	BMP-2	untreated

BMP-2 and FGF-2, alone or in combination, have no significant effect on osteocalcin gene expression in adult human trabecular bone-derived cells.

## BMP-2 expression

untreated

BMP-2			1.278
FGF-2		0.500	2.357
BMP-2 + FGF-2	0.833	0.289	0.913
	FGF-2	BMP-2	untreated

BMP-2 and FGF-2, alone or in combination, have no significant effect on BMP-2 gene expression in adult human trabecular bone-derived cells.

## BMP-4 expression

untreated

BMP-2			0.913
FGF-2		0.833	2.357
BMP-2 + FGF-2	0.500	0.289	1.278
	FGF-2	BMP-2	untreated

BMP-2 and FGF-2, alone or in combination, have no significant effect on BMP-4 gene expression in adult human trabecular bone-derived cells.

Therefore, the overall stimulation in expression of osteocalcin, BMP-2 and BMP-4 mRNA transcripts was not seen when the individual treatment groups were compared.

## 2) Compare BMP-7 and FGF-2

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
BMP-7	0.5	1.4	5.3	4.1	16.5	20.9	1.6	2.5	4.1	0.4	0.7	2.0
FGF-2	2.8			14.2			3.0			1.1		
BMP-7 +FGF-2	5.4			25.6			4.4			3.4		
critical H values	6.333			6.333			6.333			6.333		
calculated H values	3.867			<b>6.711</b>			<b>6.711</b>			3.511		



There were significant differences in the transcript expression of osteocalcin and BMP-2 following treatment with BMP-7 and FGF-2, alone or in combination, therefore for these two genes only, the null hypothesis is rejected.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### Osteocalcin expression

untreated

BMP-7			1.143
FGF-2		0.212	1.993
BMP-7 + FGF-2	0.737	0.775	2.213
	FGF-2	BMP-7	untreated

BMP-7 and FGF-2, alone or in combination, have no significant effect on osteocalcin gene expression in adult human trabecular bone-derived cells.

#### BMP-2 expression

untreated

BMP-7			1.470
FGF-2		0.212	1.831
BMP-7 + FGF-2	0.844	0.516	2.123
	FGF-2	BMP-7	untreated

BMP-7 and FGF-2, alone or in combination, have no significant effect on BMP-2 gene expression in adult human trabecular bone-derived cells.

Therefore, the overall significant stimulation in osteocalcin and BMP-2 mRNA expression was not seen when individual treatment groups were compared.

#### 3) Compare TGF-beta and FGF-2

Agent	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
untreated	1	1	1	1	1	1	1	1	1	1	1	1
TGF-beta	2.5	3.1		10.5	12.9		2.4	3.8		4.5	5.3	
FGF-2	2.8			14.2			3.0			1.1		
TGF-beta +FGF-2	2.6			19.4			2.6			4.9		
critical H values	5.833			5.833			5.833			5.833		
calculated H values	5.417			6.083			5.417			5.833		

There were significant differences in the transcript expression of osteocalcin and BMP-4 following treatment with TGF-beta and FGF-2, alone or in combination, therefore for these two genes only, the null hypothesis is rejected.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### Osteocalcin expression

untreated

TGF-beta			1.643
FGF-2		0.500	1.414
TGF-beta + FGF-2	0.833	0.289	2.008
	FGF-2	TGF-beta	untreated

BMP-7 and FGF-2, alone or in combination, have no significant effect on osteocalcin gene expression in adult human trabecular bone-derived cells.

#### BMP-4 expression

untreated

TGF-beta			0.913
FGF-2		0.667	2.122
TGF-beta + FGF-2	0	0.577	1.643
	FGF-2	TGF-beta	untreated

BMP-7 and FGF-2, alone or in combination, have no significant effect on BMP-4 gene expression in adult human trabecular bone-derived cells.

Therefore, the overall significant stimulation in osteocalcin and BMP-4 transcript levels was not seen when the individual treatment groups were compared.

#### Null hypothesis 22:

The presence of an extracellular matrix has no effect on the calcitriol-induced expression of osteoblast-related genes in human bone-derived cells

Cells	relative MSX-2 expression				relative osteocalcin expression				relative BMP-2 expression				relative BMP-4 expression			
untreated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10 nM calcitriol	1	4			1	19			0.5	2			1	2		
ascorbate-2-phosphate	1	1	1	2	1	1	1	19	1	1	2	3	1	1	1	2
ascorbate-2-phosphate + 10 nM calcitriol	1				1				3				1			
critical H value	6.386				6.386				6.386				6.386			

calculated H values	2.636	<b>6.659</b>	1.977	1.045
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The only significant effect of calcitriol and/ or ascorbate-2-phosphate was seen on osteocalcin expression, therefore, with the exception of this gene, the null hypothesis is upheld.

#### Q values

As 4 samples are being compared, the critical Q value is 2.639.

#### Osteocalcin expression

untreated

calcitriol			1.045
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ascorbate		0.609	2.023
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calcitriol+ ascorbate	2.090	1.477	0.809
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ascorbate	calcitriol	untreated
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There was no significant effect on osteocalcin expression following treatment with calcitriol and/ or ascorbate.

Therefore, the overall stimulation in osteocalcin expression levels seen following culture in ascorbate-2-phosphate and/ or calcitriol was not related to individual treatment groups.

In the following section, the expression patterns of osteoblast-related genes in subpopulations of adult human trabecular bone- and bone marrow-derived cells were examined. The subpopulations were obtained by physical separation using FACS following the labelling for the cell surface expression of Stro-1 and alkaline phosphatase antigens.

#### Null hypothesis 23:

There is no difference in osteoblast-related gene expression between subpopulations of adult human bone marrow-derived cells

#### 1) untreated cells

Subpopulation relative					relative			relative				relative			
MSX-2					osteocalcin			BMP-2				BMP-4			
expression					expression			expression				expression			
R2	0.3	0.6	1.2	1.6	0	0.2	0.7	0.2	0.2	0.4	41.5	0.1	0.3	1.9	40.3
R3	0	0	0.4	2.5	0	0.3	0.4	0	0.5	0.8	3.3	0	0.4	2.6	5.0
R4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
R5	0	0.1	0.6	1.7	0	0.9	1.1	0.5	0.7	3.1	18.8	0	0.5	0.6	14.2

critical

H values	7.235	7.000	7.235	7.235
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calculated

H values	1.163	5.154	1.483	2.796
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There is no significant difference between the gene expression levels in the four subpopulations of untreated cells, therefore the null hypothesis is upheld.

## 2) calcitriol-treated cells

Subpopulation	relative MSX-2 expression	relative osteocalcin expression	relative BMP-2 expression	relative BMP-4 expression
R2	0 2.9 3.3 11.0	0 0.1 0.7 4.5	0 0 0.3 9.1	0 0.1 0.9 3.9
R3	0 1.9 10.0 50.0	0.3 0.4 2.0 2.3	0 0.1 1.7 2.7	0.1 1.8 2.0 5.0
R4	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
R5	0 0.1 1.2 1.9	0 0.1 0.7 1.7	0 0 0.7 0.7	0 0.1 0.8 1.5

critical

H values	7.235	7.235	7.235	7.235
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calculated

H values	3.005	2.233	3.375	3.722
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There is no significant difference between the gene expression levels in the four subpopulations of calcitriol-treated cells, therefore the null hypothesis is upheld.

## 3) dexamethasone-treated cells

Subpopulation	relative MSX-2 expression	relative osteocalcin expression	relative BMP-2 expression	relative BMP-4 expression
R2	0.4 4.4 11.0	6.3 30.0	1.6 3.3 10.0 0	0.1 0.9 3.9
R3	0.3 10.0	10.0	1.3 50.0	0.8 2.5
R4	1 1 1 1	1 1 1	1 1 1 1	1 1 1 1
R5	0 0.1 1.5 1.5	0 1.7 2.0	1.5 1.6 3.2 7.0	0 3.2 7.3 12.4

critical

H values	6.874	6.244	6.874	6.874
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calculated

H values	1.769	5.600	7.954	4.368
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The only significant difference between the gene expression levels in the four subpopulations of dexamethasone-treated cells is in BMP-2 expression, therefore for this gene only, the null hypothesis is rejected.

## Q values

The critical Q value is 2.639, as there are four samples.

## BMP-2 expression

R2

R3 0.233

R4 1.927 2.465

R5 2.135 0.184 0.488

R4 R3 R2

There are no significant differences in BMP-2 expression between the four subpopulations of dexamethasone-treated adult human bone marrow-derived cells.

Therefore, the general significant effects on BMP-2 expression following culture in dexamethasone were not seen when individual fractions were compared.

## 3) cells treated with dexamethasone and calcitriol

Subpopulation	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
R2	20.5	33.0		0.9	6.0		0.4	8.9		2.0	6.8	
R3	10.0			1.1			10.0			10.0		
R4	1	1	1	1	1	1	1	1	1	1	1	1
R5	1.8	3.4	25.0	0	0.7	3.6	0.6	1.5	16.3	0.9	1.0	48.3
critical												
H values	6.244			6.244			6.244			6.244		
calculated												
H values	<b>6.311</b>			1.511			2.044			2.800		

The only significant difference between the gene expression levels in the four subpopulations of cells cultured in dexamethasone and treated with calcitriol is in MSX-2 expression, therefore for this gene only, the null hypothesis is rejected.

## Q values

The critical Q value is 2.639, as there are four samples.

## MSX-2 expression

R2

R3 0.596

R4 1.265 1.600

R5 1.641 0.105 0.933

R4 R3 R2

There are no significant differences in MSX-2 expression between the four subpopulations of adult human bone marrow-derived cells cultured in dexamethasone and treated with calcitriol.

Therefore, the overall stimulation in MSX-2 expression levels following culture in the presence of dexamethasone and calcitriol was not seen when cell fractions were compared against each other.

#### Null hypothesis 24:

There is no difference in osteoblast-related gene expression between subpopulations of adult human trabecular bone-derived cells

##### 1) untreated cells

Subpopulation relative					relative				relative				relative			
MSX-2					osteocalcin				BMP-2				BMP-4			
expression					expression				expression				expression			
R2	0	1.9	1.9	17.8	0	0.5	0.8	1.9	0	0.3	1.1	11.0	0	0.6	1.5	16.5
R3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
R4	0.3	0.7	7.3		0.3	1	7.1	0	2.0	3.6	5.0		0.4	2.5	3.0	
R5	1.4	2.5	4.6		0.1	0.7	2.8	0.5	0	6.4	6.5	0	0.2	2.5	136.8	
critical																
H values		7.038			7.038				7.038				7.038			
calculated																
H values		2.905			1.567				2.465				0.833			

There is no significant difference between the gene expression levels in the four subpopulations of untreated adult human trabecular bone-derived cells, therefore the null hypothesis is upheld.

##### 2) calcitriol-treated cells

Subpopulation	relative MSX-2 expression			relative osteocalcin expression			relative BMP-2 expression			relative BMP-4 expression		
R2	1	1	1	1	1	1	1	1	1	1	1	1
R3	2.3	2.5	0	0.3	1.0		0.8	1.0		1.0	1.4	
R4	0.4	1.7		0.5	5.0		0.6	1.7		0.7	10.0	
R5	2.8	6.0		0.8	26.5		0.9	3.7		5.4	51.0	
critical												
H values	6.333			6.333			6.333			6.333		
calculated												
H values	6.533			1.184			0.851			3.384		

There was one significant difference, between the mRNA expression levels of MSX-2, in the four subpopulations of calcitriol-treated adult human trabecular bone-derived cells, therefore for this gene only, the null hypothesis is rejected.

#### Q values

The critical Q value is 2.639, as there are four samples.

#### MSX-2 expression

R2

R3 1.400

R4 1.278 0

R5 2.008 0.730 2.200

R4 R3 R2

There are no significant differences in MSX-2 expression between the four subpopulations of calcitriol-treated adult human trabecular bone-derived cells.

Therefore, the significant effect of calcitriol on MSX-2 expression was not specifically related to changes in expression between the four fractions of cells.

#### 3) dexamethasone-treated cells

Subpopulation	relative MSX-2 expression				relative osteocalcin expression				relative BMP-2 expression				relative BMP-4 expression			
R2	0.1	1.1	1.2	1.5	1.1	1.1	2.8		0.4	3.1	6.8	11.7	1.5	2.0	4.0	17.8
R3	1.3	1.3	4.6		1.3	3.5			0.2	1.9	4.0		1.5	4.0	25.0	
R4	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
R5	0.1	2.4	3.7		0.2	0.6	2.4		0.2	0.4	1.2	3.6	0.4	0.6	1.1	16.2

critical

H values 7.038 6.727 \*\*\*\*\* \*\*\*\*\*

calculated

H values 4.551 5.166 2.132 **7.279**

\*\*\*\*\*= greater than 6.957, less than 7.235

There was one significant difference, between the mRNA expression levels of BMP-4, in the four subpopulations of dexamethasone-treated adult human trabecular bone-derived cells, therefore for this gene only, the null hypothesis is rejected.

#### Q values

The critical Q value is 2.639, as there are four samples.

## BMP-4 expression

R2

R3 0.196

R4 2.099 2.056

R5 0.395 1.733 1.660

R4 R3 R2

There are no significant differences in BMP-4 expression between the four subpopulations of dexamethasone-treated adult human trabecular bone-derived cells.

Therefore, the overall significant effects of dexamethasone on the expression of BMP-4 transcripts are not seen when the individual fractions are compared.

## 4) cells treated with calcitriol and dexamethasone

Subpopulation	relative MSX-2 expression		relative osteocalcin expression		relative BMP-2 expression		relative BMP-4 expression	
R2	2.3	4.9	5.8	8.9	8.7	10.7	9.3	10.9
R3	2.9		1.6		1.7		6.4	
R4	1	1	1	1	1	1	1	1
R5	2.4	14.7	3.3	11.4	2.0	6.7	6.4	6.9
critical								
H values	5.679		5.679		5.679		5.679	
calculated								
H values	3.964		4.821		<b>5.679</b>		5.439	

There was one significant difference, between the mRNA expression levels of BMP-2, in the four subpopulations of adult human trabecular bone-derived cells treated with calcitriol and dexamethasone, therefore for this gene only, the null hypothesis is rejected.

## Q values

The critical Q value is 2.639, as there are four samples.

## BMP-2 expression

R2

R3 1.323

R4 0.567 2.315

R5 1.389 0.694 0.926

R4 R3 R2

There are no significant differences in BMP-2 expression between the four subpopulations of adult human trabecular bone-derived cells treated with calcitriol and dexamethasone.



Therefore, the overall significant effects on BMP-2 transcript expression of treatment with calcitriol and dexamethasone in combination are not seen when the individual cell fractions are compared.

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